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The use of brewers condensed solubles in bivalve mariculture

David S. Gussman
College of William and Mary - Virginia Institute of Marine Science

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Gussman, David S., Ph.D.
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U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

THE USE OF BREWERS CONDENSED SOLUBLES IN BIVALVE MARICULTURE

A Dissertation

Presented to

The Faculty of the School of Marine Science

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Doctor of Philosophy

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by

David S. Guseman

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APPROVAL SHEET

This dissertation is submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

David S. Gussman

Approved, May 1987

Howard I. Kator, Ph.D.
Committee Co-Chairman

Morris H. Roberts, Jr. Ph.D.
Committee Co-Chairman

William B. Dunstan, Ph.D.
Old Dominion University
Norfolk, Virginia

Dexter S. Haven, M.S.

Leonard W. Haas, Ph.D.

Bruce J. Neilson, Ph.D.

THE USE OF BREWERS CONDENSED SOLUBLES IN BIVALVE MARICULTURE

ABSTRACT

Brewers Condensed Solubles (BCS), a by-product of the brewing industry, was evaluated as a nutrient source for rearing juvenile oysters (Crassostrea virginica) and clams (Mercenaria mercenaria). The BCS was used to culture bacteria which were fed to colorless flagellates which were in turn fed to the oysters and clams. The overall growth efficiency of oysters on BCS was 473 mg of oyster (whole weight) per g of BCS (dry weight).

Fourteen isolates representing nine genera of bacteria were isolated from BCS enrichment cultures. Specific growth rates of the isolates at 24°C on a BCS medium ranged from 0.48 h^{-1} to 0.11 h^{-1}. The conversion of BCS to bacterial biomass was examined for four isolates with supplements of 0, 38, 76, and 152 mg/l of ammonium sulfate. The largest bacterial biomass (127 mg/g BCS) was obtained with Pseudomonas marina. The largest bacterial biomass when averaged over all bacterial isolates was obtained with 38 mg/l of ammonium sulfate. Yields of P. marina on BCS ranged from 27.4% for no ammonium sulfate addition to 38.5% with 38 mg/l of ammonium sulfate.

The effects of dissolved nutrients, salinity, temperature, shaking, bacterial concentration, and bacterial species on the growth rates of five species of colorless flagellates were examined. None of the

colorless flagellates could be raised on the dissolved nutrients in BCS, all required a bacterial diet. Shaking, salinity, and the bacterial isolate used as food had little effect on the flagellate growth rates. Temperature and bacterial concentration had pronounced effects. The greatest growth rates were recorded at temperatures between 21 and 26°C. Growth rates increased with increasing bacterial concentration in a manner suggestive of Michaelis-Henten kinetics. Maximum specific growth rates ranged from 0.11 h^{-1} to 0.16 h^{-1}. Yields of colorless flagellates growing on bacteria ranged from 0.30 to 0.42.

The growth of oysters and clams fed colorless flagellates, BCS enrichment cultures, and bacteria was compared to the growth of starved controls and animals fed Tetraselmis suecica. Paraphysomonas vestita was the only species of colorless flagellate to consistently result in growth greater than the starved control. The BCS enrichment culture varied greatly in its nutritional value. The average oyster growth on P. vestita was 55% of the growth obtained with T. suecica. Oysters fed combinations of T. suecica and P. vestita did not grow as rapidly as on a pure diet of T. suecica. No growth occurred when oysters and clams were fed a purely bacterial diet.

David S. Gussman

School of Marine Science

The College of William and Mary in Virginia

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The effects of dissolved nutrients, salinity, temperature, shaking, bacterial concentration, and bacterial species on the growth rates of five species of colorless flagellates were examined. The five species studied included Paraphysomonas vestita, an unidentified chrysomonad, two bodonids, and a choanoflagellate. None of the colorless flagellates could be raised on the dissolved nutrients in BCS, all required a bacterial diet. Shaking, salinity, and the bacterial isolate used as food had little effect on the flagellate growth rates. Temperature and bacterial concentration had pronounced effects. The greatest growth rates were recorded at temperatures between 21 and 26°C. Growth rates increased with increasing bacterial concentration in a manner suggestive of Michaelis-Menten kinetics. Maximum specific growth rates ranged from 0.11 h^{-1} to 0.16 h^{-1}. Yields of colorless flagellates growing on bacteria ranged from 0.30 to 0.42.

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THE USE OF BREWERS CONDENSED SOLUBLES IN BIVALVE MARICULTURE

SECTION I.

Introduction

Waste water aquaculture is a term used to describe the process by which aquatic organisms are raised for food on nutrients derived from a waste effluent. A well designed waste water aquaculture system can dispose of waste nutrients without stressing the environment while producing a food for man. By combining these two processes into one system, significant savings in energy and costs may be realized. The present study examined the feasibility of a waste water aquaculture system using brewery waste for raising the American oyster (Crassostrea virginica) and the hard clam (Mercenaria mercenaria). Bacteria and protozoa were first cultured on the brewery waste effluents and were then tested as diets for the bivalves.

Most conventional forms of waste treatment, e.g. activated sludge, trickling filter, rotating biological contactors, and oxidation ponds, use microorganisms to oxidize organic wastes. Nutrients in some of these systems are removed and recycled in the form of sludge, but most often are released into the aquatic environment in a more oxidized form. Large quantities of nutrients released into a body of water result in eutrophication, a condition which results in a large increase in the biomass of algae and other microorganisms. Respiration by the increased microbial biomass depletes oxygen from the water and the resulting anaerobic conditions

are fatal to fish, bivalves, and other aquatic life. High nutrient concentrations may themselves be toxic to aquatic animals. Excess nutrients are eventually swept out to sea or sequestered in sediments. Waste water aquaculture can be considered to be "controlled eutrophication" (Ryther et al., 1976). The waste nutrients are used to culture selected microorganisms under carefully regulated conditions and then the microorganisms are harvested and fed to animals which can serve as human foods. The oxidized nutrients are thus recycled rather then released into the aquatic environment.

In designing a waste water aquaculture system, careful attention must be given to the chemical and physical characteristics of the waste. Of particular importance will be the concentrations of primary nutrients, e.g. carbon, nitrogen, and phosphorus, for these will determine the amount of microbial biomass which can be produced and thus ultimately the amount of any desired food organisms. The ratios of nutrients will determine if supplementation with inorganic nutrients is required to maximize the production of biomass. The oxidation state of the nutrients, which reflects the energy available for growth, is another important factor. Physical characteristics to be considered include the degree of dissolution and the size distribution of particulates in a waste. Wastes low in nutrients, extremely inert, or toxic may be undesirable. Both industrial and domestic wastes may be suitable for waste water aquaculture.

Animal and human wastes, and agricultural residues can all be used as sources of nutrients for waste water aquaculture. For centuries, waste water aquaculture was practiced on a small scale in the form of fish ponds that were fertilized with human nightsoil.

Human wastes have been used in a variety of more intensive aquaculture projects in recent years. Raw sewage can be used to fertilize ponds and lagoons for fish rearing without any pretreatment (Carpenter et al., 1976; Henderson, 1979; Avelallement and Held, 1980). Aquaculture can also be employed as a form of tertiary waste treatment, using secondary treated sewage effluents as a source of nutrients (Ryther, 1976; Allen et al., 1981; Kawasaki et al., 1982). Sewage sludge, with a without composting, can also be used as a nutrient source for fertilizing ponds (Banerjee and Srinivansan, 1983; Polprasert et al., 1984). Cattle (Schroeder and Hepher, 1976), swine (Buck, Bauer, and Rose, 1978; Watson, 1985), and poultry wastes (Ling, 1977; Griffin et al, 1980; Burns and Stickney, 1980) have all been used as nutrients in mariculture projects. Many agricultural wastes and residues (i.e. cocoa hulls, cotton seed, peanut shells, rice bran, sugar cane fibre) have been examined as nutrient sources for aquaculture (Bardach, 1978) with mixed results. Most studies on these wastes tested them as feed supplements for fish with little treatment beyond grinding and pelletizing.

Few industrial wastes have been examined for use in aquaculture compared to the number of projects involving human and animal wastes even though many industrial wastes, particularly those of the food and beverage industries, have high organic contents and are not highly toxic. Discarded animal parts from food processing industries have been examined for use in aquaculture in a limited number of studies. Chicken viscera have been used as a food supplement for raising salmon (Market, 1977), scraps from clam processing can be used in trout rations (Goodrich et al., 1984), and wastes from fish and shellfish

processing have been used to raise catfish (Dean et al., 1982). Abbatoir wastes and dairy wastes have been used to raise Euglena gracilis which were then fed to zooplankton and fish (Waygood et al., 1980). Other food industry wastes used for aquaculture include cannery effluents used to raise photosynthetic bacteria and algae which are fed to tilapia (Gaigher et al., 1980), sugar beet factory effluents used to fertilize carp ponds (Thorslund, 1971), and effluents from a palm oil refinery used to culture Chlorella sp. for zooplankton rearing.

While brewery wastes have never been used for raising marine bivalves, studies have examined their use and that of related fermentation industry wastes for other types of aquaculture. Single cell protein from microorganisms raised on brewery wastes have been incorporated into trout rations (Windell et al., 1974). Ponds fertilized with brewery wastes have resulted in yields of tilapia of 2,500 kg/ha (Nugent, 1978). Distillation slops from rum distilleries and spent beer from pharmaceutical fermentations have been used to raise tilapia (Pagan-Font, et al., 1978) as have distillery wastes from ethanol production plants (Behrends et al., 1985). All these studies have cultured fish, and most have chosen tilapia, which can feed on phytoplankton, as the cultured animal. The one reported attempt at raising an aquatic food organism (fresh water prawns) on distillery wastes was not successful (Behrends et al., 1985).

Most wastes cannot be fed directly to animals consumed by man. At least one and usually several microbial intermediates are required in the food chain. Waste water aquaculture systems can be characterized by the number of such stages, the types of

microorganisms involved, and the source of energy used for the microbial growth. Many waste water aquaculture schemes have used bacteria to oxidize organic compounds in a first stage. Microalgae are raised on the oxidized nutrients in a second stage and the microalgae are fed to the food organism or another intermediate herbivore in a third stage. The first stage is dependent on energy present in the waste while the second stage requires the input of energy in the form of light, and the third stage is dependent on the energy present in the algae. The first and third stages involve heterotrophic processes while the second step involves an autotrophic process. The inclusion of an autotrophic process imposes a number of restrictions on the overall aquaculture system and may act as a "bottleneck".

The production of microalgae requires light which may be provided either artificially by lamps or naturally by sunlight. The cost of electricity makes algal culture under lamps prohibitively expensive for commercial ventures. Sunlight is a less expensive source of illumination but is not available at night and is greatly reduced on cloudy days. Severe restrictions on the sizes and shapes of algal culture vessels result from the requirement for sufficient light penetration. Culture vessels used with artificial lights rarely exceed one meter in the dimension of light penetration. Outdoor ponds must be shallow as well to allow light penetration. Since covers reduce light penetration by absorbing and reflecting light, ponds are frequently open to the air, which leads to contamination problems.

An aquaculture system that uses only heterotrophic processes and thus obviates the need for high intensity light was a goal of the

present study. Bacteria and protozoa, both of which can be raised in the dark, were cultured on brewery wastes. No attempt was made to raise algae. A waste water aquaculture system that uses only heterotrophic processes should, in theory, be more efficient in the utilization of both energy and space than those that incorporate a photosynthetic stage.

Breweries lend themselves to waste water aquaculture for several reasons. Brewery waste effluents contain high concentrations of nutrients which are easily degradable. Typical BODs are between 1,000 mg/l and 3,000 mg/l (Schwartz and Jones, 1972). At present, brewery waste effluents are usually treated at municipal treatment plants where they are diluted with domestic waste waters and treated by an activated sludge process (Schwartz and Jones, 1972). Sludge bulking is frequently a problem due in part to the high proportion of easily assimilated carbohydrates in the effluent (Schwartz and Popowchak, 1980). Easily assimilated carbohydrates, however, would be an advantage in a waste water aquaculture system. Large quantities of waste effluents are generated by the brewing process, usually about ten barrels of waste water for each barrel of beer (Schwartz and Jones, 1972). At the Anheuser-Busch brewery in Williamsburg, VA, 100,000 barrels of beer are produced per week resulting in three to four million gallons of waste water per day. Breweries generate waste effluents continuously throughout the year on a regular schedule, an important consideration if an aquaculture treatment system is to be operated year round. Many food processing industry wastes that might be favorable for aquaculture are only available seasonally. The composition of brewery waste effluents remains relatively constant

over time. Microbial production problems could result if wastes fluctuated greatly in composition. Breweries are also heavy users of energy; an average of 390,000 BTUs are required to produce a barrel of beer (Keenan and Kormi, 1981). It might be possible to use some of the waste heat in the mariculture operation.

Several waste water aquaculture systems have used effluents from municipal waste treatment plants (Ryther et al., 1972; Shelef et al., 1980; Allen and Carpenter, 1977). Heavy metals, insecticides, herbicides, and hydrocarbons can all occur in the influent of a municipal treatment plant with potentially harmful effects on the organisms being cultured. Breweries do not have this problem because they control what is going into waste streams. Every compound used in a brewery must meet standards set by the FDA for the beverage industry. Waste water aquaculture systems that use treated human or animal wastes must also address the potential for accumulation of pathogenic bacteria and viruses in the cultured animals (Mann et al., 1979). Human pathogens are extremely rare in breweries because the fermentation process produces conditions (low pH, high CO_2, and high alcohol) which are unfavorable to their growth.

Bivalves, which can be raised on microorganisms, feed lower in the food chain than many species of fish, which require zooplankton for food. The elimination of a trophic level increases the amount of human food that can be produced per volume of waste treated and simplifies the operation of the aquaculture system. For these reasons, Bivalves have been examined in several waste water aquaculture systems. Secondary treated sewage effluent mixed with

seawater was used to raise marine phytoplankton which was fed to bivalves in a large pilot project at the Woods Hole Oceanographic Institute (Ryther, 1976). Bivalves evaluated for growth in the system included Crassostrea virginica, Crassostrea gigas, Ostrea edulis, Mercenaria mercenaira, Tapes japonica, Mytilus edulis (Mann and Ryther, 1977), and Argopecten irradians (Mann and Taylor, 1981). Another aquaculture project examined the use of fish processing waste water to raise M. edulis. The waste water was found to be a poor food for M. edulis but some growth occurred when it was mixed with phytoplankton and silt (Murken, 1975).

A less intensive form of waste water aquaculture is practiced in Taiwan where hard clams (Meretrix lusoria) raised in coastal ponds reach market size in 6-8 months instead of one year when the ponds are fertilized with fish meal and fish solubles (Chen, 1984). Waste water aquaculture schemes with bivalves are not limited to estuarine and marine environments, freshwater clams (i.e. Corbicula fluminea) can be cultured as well (Greer and Ziebell, 1972).

Economic considerations make the Eastern oyster Crassostrea virginica and the hard clam Mercenaria mercenaria prime candidates for waste water aquaculture. Natural stocks of both bivalve have declined dramatically in recent years and no reversal in the trend is predicted for the future (U.S. Dept. of Commerce, 1977). The demand for oysters is believed to be elastic; therefore an increase in production might increase the market for oysters (Haven, et al., 1978). The high unit market value for oysters and clams suggests that a relatively small facility and concomittant investment might have a reasonable chance of success (MIT Sea Grant, 1977).

Microorganisms, raised on brewery wastes and suitable for bivalve consumption, could be used as food in a bivalve hatchery. The technology for raising bivalves in a hatchery is well developed (DuPuy, et al., 1977; Castagna and Kraeuter, 1981; Bolton, 1982). Bivalves spawned and set in hatcheries have more uniform size and shape which increase their market value. Hatcheries may select for strains of bivalves that exhibit greater growth rates, higher yields of edible meats, and a more efficient utilization of nutrients than natural populations. Disease resistance and improved flavor are other possible genetic improvements that might be developed in hatchery reared animals. A number of privately owned commercial hatcheries are demonstrating that it is indeed a financially viable business.

Hatcheries typically raise oysters to a juvenile size and then transfer them to a "grow out" operation or sell them to growers who raise them to market size on natural seedbeds. Many parts of an estuary will support good growth of oysters but do not have sufficient natural recruitment to maintain populations due to overharvesting, pollution, or a variety of environmental factors (currents, poor substrate, salinity). Planted oysters may grow well in environments where natural spatfall and survival of spat is poor. The survival of juvenile oysters planted on seedbeds increases dramatically with small increases in size. Hatcheries, however, do not raise oysters beyond the early juvenile stage because it becomes prohibitively expensive to provide juvenile oysters with the large amounts of food which they require. If brewery wastes can be used to provide a food for use in oyster hatcheries, large juveniles, which command better prices from growers, may be produced more economically. It may even be feasible

to raise large numbers of market sized oysters under controlled conditions.

Three schemes for raising juvenile bivalves on brewery waste were considered in the present study. The first was to raise bivalves on bacteria that were cultured on brewery wastes. The second was to raise bivalves on protozoa that were cultured on brewery wastes. The third was to raise bivalves on protozoa that were cultured on bacteria which were raised on brewery wastes. The feeding of brewery wastes directly to bivalves was not tested.

The descriptions of the experiments are organized into three parts, Sections II, III, and IV. Section II details the properties of brewery wastes and describes the composition of Brewer's Condensed Solubles (BCS), the brewery by-product which was used for the remainder of the study. Section II also describes the isolation of estuarine bacteria capable of growth on BCS, characterization of these bacteria, and information on bacterial growth rates and yields when cultured on a BCS medium. Section III describes the culture of protozoan flagellates on BCS and bacteria cultured on BCS. Section IV presents the experiments testing the growth of oysters and clams fed bacterial and protozoan diets. Finally section V integrates the results from the previous sections to describe how waste water aquaculture using BCS would affect the operation of a bivalve hatchery.

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SECTION II

Analysis of Brewers Condensed Solubles and Growth of Bacteria

INTRODUCTION

Brewers condensed solubles (BCS) is a by-product of brewery waste treatment processes designed to reduce the BOD of brewery effluent prior to treatment at a conventional municipal sewage treatment plant. Without this pretreatment, full strength brewery effluents often result in sludge bulking problems at the treatment plant if insufficient domestic wastes are available for dilution. BCS, which is produced by a series of high temperature, low pressure evaporators, contains the nutrients equivalent to a much larger volume of untreated brewery effluent. Waste streams treated to produce BCS include spent grain liquor, trub press liquor, spent yeast, and beer spilled in packaging operations. BCS is currently being marketed as a food supplement for the dairy cattle and broiler chicken industries in competition with inexpensive grain products.

Investigations of BCS as a nutrient source for production of marine bacterial biomass are described in this section. Other industrial by-products such as beet, corn, and wood molasses have been used commercially for microbial biomass production. Studies with these by-products have demonstrated that concentrations of protein and carbohydrate, pH, and solids content can influence the effectiveness of these substrates for microbial growth (Sobkowiscz, 1972). Nitrogen

content and the C:N ratio are important variables for the production of microbial biomass (Litchfield, 1979). Many by-products must be supplemented with nitrogen in order to maximize the production of microbial biomass. The above mentioned properties were investigated for BCS to determine its suitability as a substrate for bacterial growth.

To evaluate the use of BCS as a growth medium, bacteria capable of growth on BCS were isolated from the York River and characterized. To increase the number of isolates, an enrichment procedure was performed in March, May, and August because the microbial flora of an estuary will show large changes in species composition over this period of time (Sieburth, 1967). Selected characteristics of the isolates were examined which will influence the production of bacterial biomass on BCS. Among the characteristics were growth rate, type of metabolism, biomass conversion ratio and yield.

MATERIALS AND METHODS

Brewers Condensed Solubles

BCS was obtained from the Anheuser-Busch brewery in Williamsburg, VA. Samples were collected in sterile one gallon containers and stored at 5°C. BCS was characterized by measuring total solids, soluble solids, proximate carbohydrate content, and total protein.

The total solids content of BCS was determined by drying triplicate 1.0 gram (wet weight) samples in tared aluminum drying pans for 24 hours at 80°C, a temperature that minimized loss of volatile compounds. To measure soluble solids, 200 g of BCS (wet weight) was mixed with 400 ml of distilled water followed by centrifugation at 10,000 G for 20 minutes. The supernatant was filtered through a 0.45 um GA-6 cellulose triacetate membrane filter (Gelman Sciences, Inc.). The filtrate was designated as brewers condensed solubles filtrate (BCSF). Ten-ml aliquots of BCSF were transferred to tared aluminum pans and dried at 80°C for 24 hours, cooled in a dessicator and reweighed. The suspended solids content was not measured directly, but was calculated as the difference between total solids and soluble solids.

The proximate carbohydrate content of BCS and BCSF was determined by the anthrone reagent method (Allen, 1974). Total protein was determined by the biuret method described in Herbert et al. (1971). Absorbance for protein and carbohydrate analyses was determined using a Spectronic 70 spectrophotometer (Bausch and Lomb, Rochester, N.Y.).

Total organic carbon (TOC) content of BCS and BCSF was measured on a Total Organic Carbon Analyzer (Beckman Model 915B), using the acid-sparge method. Samples were acidified with 1.0 N HCl to pH 2 and sparged with CO_2-free nitrogen gas for 10 minutes. The carbon analyzer was calibrated with anhydrous potassium bithalate (Model 915B Operating Manual, Beckman, 1978) and the full scale reading was adjusted to equal 40 ppm (weight/volume) organic carbon. Response of the model 915B was linear in the range of 0 to 40 ppm. Samples were diluted with CO_2-free distilled water to yield readings within the calibration range. Three 40-ul aliquots of each sample were analyzed and the results averaged.

Bacterial isolation, growth, and identification

In order to isolate strains of bacteria which could use BCSF as a nutrient source, an enrichment medium was prepared with 2.0 l of filtered (5.0 um) York River water containing 1.0 g (wet wgt) of BCSF/l. The medium was prepared in a Fernbach flask plugged with a cotton stopper. The cultures were aerated with compressed air filtered through glass wool via a sintered glass disc diffuser. Every day for a week, 1.0 l of culture was removed after vigorous shaking and replaced with 1.0 l of sterile, BCSF enriched (1.0 g/l) York River water.

On the eighth day serial dilutions of the culture were plated onto BCSF medium. BCSF medium contained 1.0 g BCSF, 0.01 g ferric citrate, and 0.1 g sodium glycerol phosphate in a liter of seawater. The pH was adjusted to 7.6 and the salinity adjusted to 17°/oo. Plates were incubated at 24°C for 48 h. Colonies were isolated from the agar surface, suspended in sterile BCSF broth and streaked onto fresh BCSF agar. An effort was made to select colonies which appeared different.

Bacteria were isolated by this procedure in March, May, and August. Isolates were subsequently grown on BCSF agar slants and in a BCSF broth and were subcultured every two weeks.

Colony characteristics were described from isolates grown for 48 h on BCSF agar plates. Motility, size, shape, and cell arrangement were described from wet mounts of log phase (18 to 24 h after inoculation) BCSF broth cultures. Log phase cells was stained using the Hucker modification of the Gram stain (Doetsch, 1981). Gram positive isolates were tested for catalase activity by placing one drop of 15% H_2O_2 directly on a 48 h old BCSF agar colony.

The classification scheme of Shewan (1963) was used for taxonomic analysis of gram negative isolates. The presence of cytochrome oxidase in 48 h old colonies was determined by Kovacs oxidase test (Kovacs 1956) using a commercially prepared Kovacs reagent (Cepti Seal, Marion Scientific). Growth on glucose, maltose, fructose, sucrose, and lactose was determined using Liefson's marine oxidation fermentation (MOF) medium. The MOF medium was prepared with 1% concentrations of the various carbohydrates. Cultures in log growth phase were inoculated into the MOF media and fermentation tubes were sealed with Vaspar. Growth, color changes, and gas production were recorded at 1, 7, and 14 day intervals. Sensitivity to chloramphenicol (30 ug) and penicillin G (2 i.u.) were determined using Sensi-Discs (BBL) on BCSF spread plates of the isolates. Sensitivity to 0/129 pteridine was tested on the same plate using discs containing 400 ug pteridine per disc. Zones of inhibition were measured at 48 h.

The growth of fourteen BCS isolates and Pseudomonas marina, a stock isolate from the VIMS Bacteriology Culture Collection, was examined in

batch cultures. Each isolate was subcultured from a BCSF agar slant into 10.0 ml of BCSF broth 24 h prior to a growth experiment. One ml of 24 hour broth was used to inoculate 100 ml of sterile, filtered (0.2 um), YRW and 0.1 g of BCSF in a 250 ml Erlenmeyer flask. The salinity of the York River water was 19°/oo in all bacterial growth experiments. Flasks were plugged with cotton stoppers and kept on a Junior Orbit rotary shaker table (Lab-Line Instrument Co., Melrose Park, IL.) at 100 rpm in a 24°C incubator. Absorbance of the cultures was measured at 660 nm using one cm cells at 0, 4, 8, 12, 15, and 24 hours. Each isolate was cultured in triplicate. Specific growth rates were calculated from the absorbance readings during the exponential phase of growth. The linear relationship between absorbance and time was determined by a least squares regression model.

The dry weight conversion ratio of BCSF to bacterial biomass was determined with and without the addition of supplemental nitrogen. The BCSF used in these experiments had a C:N ratio of 53.5:1. To lower this ratio to 10:1 required the addition of 16.1 mg of nitrogen per g BCSF, which is the nitrogen equivalent of 76 mg ammonium sulfate. Supplemental nitrogen of 38, 76, and 152 mg of ammonium sulfate per g BCSF was used.

The dry weight conversion ratio was determined for four isolates and for the stock culture of Pseudomonas marina. Batch cultures, as previously described, were used except they contained 200 ml of broth (1.0 g BCSF/l) in a 500 ml flask. Dry weight determinations were performed on cells harvested by centrifugation (10,000 g for 20 minutes). Harvested cells were resuspended in a 0.9% (w/v) solution of ammonium formate and centrifuged a second time. The remaining pellet

was rinsed with distilled water into a tared aluminum pan and dried for 24 h at 90° C. Pans were weighed to the nearest 0.1 mg. Two-way ANOVA was used to compare the effects of supplemental nitrogen and bacterial isolate on the bacterial biomass produced. Significant differences among the bacterial isolates and among the nitrogen supplements were determined by the Student-Newman-Keuls multiple range test (Zar, 1974).

Bacterial yields and carbon conversion ratios were calculated on the basis of organic carbon. Organic carbon was measured at each nitrogen supplement concentration for P. marina. For the other four species, organic carbon was measured only for unsupplemented treatments. The concentration of organic carbon in the media was measured initially after inoculation, prior to the cells being harvested, and in the media after the cells were harvested. Respired organic carbon was considered to be the difference between the initial organic carbon content and the organic carbon content prior to centrifugation. Sterile flasks with BCSF were used to correct for the loss of volatile constituents in the BCSF. Bacterial biomass was calculated as the difference between the organic carbon measurement prior to centrifugation and after centrifugation. Carbon conversion ratios (mg/g) were calculated by dividing the bacterial biomass by the initial amount of organic carbon added to each flask (0.198 g). Bacterial yields were calculated by dividing the bacterial biomass by the sum of the biomass plus the respired organic carbon and multiplying by 100. Carbon conversion efficiencies and yields of the bacterial isolates were compared by one-way ANOVA. Significant differences were determined by the Student-Newman-Keuls multiple range test.

RESULTS

Brewers Condensed Solubles

BCS is composed primarily of soluble solids, which accounted for 91.4% to 96.6% of the total solids in the six samples which were analyzed (Table II.1). The total solids content of the six BCS samples averaged 558 mg/g, while the soluble solids averaged 528 mg/g and suspended solids averaged 29 mg/g. Carbohydrate was the predominant solids component in both BCS and BCSF accounting for 72.8% (406 mg/g) and 74.4% (393 mg/g) of the solids (Table II.2). Protein represented 8.6% (48 mg/g) of the solids in BCS and 5.3% (27.8 mg/g) in BCSF. Protein and carbohydrate contents of the suspended solids were assumed to be the difference between the BCS and BCSF values.

The relative abundances of the nutrients in BCSF were expressed as ratios of carbohydrate:protein, C:N, and TOC:N (Table II.3). The C:N ratio was derived from the measured quantities of carbohydrate and protein in Table II.2. Total quantity of carbon was calculated as the theoretical sum of carbohydrate plus protein carbon. Theoretical carbon content was calculated as follows: carbohydrate as 40% of the dry weight using the formula $C_nH_{2n}O_n$ for carbohydrate; protein as 27.8% of the dry weight based on the formula $C_{16}H_{24}O_5N_4$. Theoretical nitrogen content was 19.1% of the dry weight. Ratios of TOC:N were calculated from the measured TOC values and the percentage of nitrogen in protein. The quantity of carbon accounted for in carbohydrate and protein was compared with the measured TOC in column five.

Table II.1
Total, Soluble, and Suspended Solids in Six Samples of BCS

sample	total solids (mg/g)	soluble solids (mg/g)	suspended solids (mg/g)	percent soluble solids
1	624	600	24	96.2
2	574	533	41	92.8
3	502	485	17	96.6
4	537	514	23	95.7
5	568	543	25	95.6
6	545	498	47	91.4
mean	558	528	29	94.7
standard deviation	41	40	11	2.1

Table II.2

Proximate Analysis of Six Samples of BCS and BCSF

sample	carbohydrate (mg/g)		protein (mg/g)		total organic carbon (mg/g)	
	BCS	BCSF	BCS	BCSF	BCS	BCSF
1	430	424	64.2	40.2	265	251
2	413	393	52.2	30.4	223	216
3	356	347	39.1	20.8	199	192
4	402	384	42.4	21.1	213	204
5	455	447	52.8	34.8	236	224
6	383	362	37.1	19.4	213	198
mean	406	393	48.0	27.8	225	214
standard deviation	35	38	10.3	8.6	23	22

Table II.3

Ratios of Carbohydrate:Protein, C:N, TOC:N, and C:TOC in Six Samples of BCSF

sample	carbohydrate:protein	C:N	TOC:N	C:TOC (%)
1	10.5	24.8	32.7	76.0
2	12.9	29.8	37.2	80.2
3	16.7	37.7	48.3	77.9
4	18.2	40.9	50.6	80.7
5	12.8	29.6	33.7	88.0
6	18.6	41.8	53.4	78.3
mean	14.9	34.1	42.6	80.2
standard deviation	3.3	6.9	9.1	4.2

Bacteria

Fourteen isolates representing nine genera of bacteria were obtained from three BCS enrichment cultures (Table II.4). Colony characteristics and cellular morphology of these isolates are listed in Appendix 1, biochemical characteristics are listed in Table II.4. Only two genera, Alcaligenes (3 isolates) and Flavobacter (2 isolates), were found in enrichments made in more than one month (Table II.5). Four genera, Aeromonas, Alcaligenes, Flavobacter and Pseudomonas II were represented by more than one isolate. Other bacterial isolates may have been present in the enrichment culture since only noticeably different colonies were isolated from each agar plate. Of these colonies, only those readily subcultured were identified. The four to six isolates from each enrichment culture were not all present in equal abundance. Usually one or two colony types predominated on a BCSF plate. The remainder were rare but were isolated if they had an unusual characteristic which made them stand out, such as pigmentation or shape of margin.

All isolates were capable of growth on maltose and sucrose, the major disaccharides in BCS, as well as on glucose (Table II.6), and all but one isolate (5013) were able to grow on fructose. Four of the fourteen isolates were unable to grow on lactose. Ten of the isolates, comprising five genera, exhibited only oxidative metabolism in their growth on the carbohydrates examined. The remaining four isolates exhibited fermentative metabolism and were able to utilize all five carbohydrates tested. Vibrio (8124) and Aeromonas (8121, 8123), the identified fermentative genera, were both isolated in August (Table

Table II.4

Assigned Taxa of BCS Grown Isolates Based On Selected Characteristics

isolate	taxon	Gram stain	Kovacs oxidase	catalase	Penicillin(G) 2 i.u.	Chloramphenicol 30 ug	Pteridine 400 ug
3231	Alcaligenes	-	+	+	+	+	+
3233	Pseudomonas group IV	-	+	+	+	+	+
3234	Pseudomonas group 2	-	+	+	-	+	+
3235	Pseudomonas group II	-	+	+	+	+	+
3236	Flavobacter	-	+	+	+	+	+
5012	unidentified rod	+	-	+	+	+	+
5013	Micrococcus	+	-	+	+	+	+
5015	Flavobacter	-	+	+	+	+	+
8121	Aeromonas	-	+	+	-	+	-
8122	Acinetobacter	-	-	+	+	+	+
8123	Aeromonas	-	+	+	-	+	-
8124	Vibrio	-	+	+	-	+	+
8125	Alcaligenes	-	+	+	+	+	+
8126	Alcaligenes	-	+	+	+	+	+

Table II.5

Dominant Marine Bacteria Isolated from Enrichment Cultures in March, May, and August of 1982

Taxonomic Group	Month		
	March	May	August
Acinetobacter			1
Aeromonas			2
Alcaligenes	1		2
Flavobacter	1	1	
Pseudomonas II	2		
Pseudomonas IV	1		
Micrococcus		1	
Vibrio			1
Gram positive bacilli		1	
total isolates	5	3	6

Table II.6

Utilization of Glucose, Maltose, Fructose, Sucrose, and Lactose in MOF Medium by Fourteen Bacterial Strains Capable of Growth on BCSF

isolate	glucose	maltose	fructose	sucrose	lactose
3231	O	O	O	O	O
3233	O	O	O	O	O
3234	O	O	O	O	-
3235	O	O	O	O	-
3236	O	O	O	O	O
5012	F	F	F	F	F
5013	O	O	-	O	-
5015	O	O	O	O	O
8121	F	F	F	F	F
8122	O	O	O	O	O
8123	F	F	F	F	F
8124	F	F	F	F	F
8125	O	O	O	O	O
8126	O	O	O	O	-

Key: O = oxidative, F = fermentative, (-) = no reaction

II.5). An unidentified gram positive rod (5012), isolated in May, was the fourth fermentative isolate. All March isolates were oxidative.

Specific growth rates of the isolates on BCSF medium (Table II.7) ranged from 0.48 h^{-1} for Pseudomonas sp. (3234) to 0.11 h^{-1}, for another isolate of Pseudomonas (3235). The fast growing isolates generally remained in exponential phase for shorter intervals than slow growing isolates. Pseudomonas sp. (3234), P. marina, and Acinetobacter sp. (8122), the first, second, and fourth fastest growing isolates remained in exponential phase about six hours. The slowest growing isolate, Pseudomonas sp. (3234) had an exponential growth phase lasting 18 hours as did Aeromonas sp. (8121), also a slow growing isolate. Measurable turbidity was not recorded with these isolates at six hours as occurred with all other isolates. Micrococcus sp. (5013) remained in exponential phase for at least 21 hours, the longest interval of any isolate. Most isolates remained in exponential phase for 15 h.

No obvious relationship between specific growth rate of an isolate and the month in which it was isolated was observed (Table II.5). The highest and lowest growth rates were both recorded with March isolates and the second highest and second lowest were recorded with August isolates. The fermentative isolates as a group did not differ in growth rates from the oxidative isolates. One Aeromonas isolate (8123) had a relatively high specific growth rate yet the Vibrio (8124) isolate and the unidentified gram positive rod (5012) were among the slowest growers.

The dry weight conversion ratio was determined for five isolates grown on BCSF (Table II.8a). Three isolates were chosen because they had the highest specific growth rates recorded, Pseudomonas sp. (3234),

Table II.7

Growth Rates of Bacterial Isolates Cultured on BCSF

isolate	taxon	specific growth rate (h^{-1})	doubling time (h)	correlation coefficient	interval of exponential phase (h)
3231	*Alcaligenes*	.169	4.1	.970	3 - 15
3233	*Pseudomonas* group 4	.169	4.09	.988	3 - 15
3234	*Pseudomonas* 1 group 2	.478	1.45	.996	3 - 9
3235	*Pseudomonas* 2 group 2	.109	6.35	.929	6 - 24
3236	*Flavobacter*	.126	5.50	.989	3 - 15
5012	unidentified rod	.155	4.46	.985	3 - 12
5013	*Micrococcus*	.206	3.36	.989	3 - 24
5015	*Flavobacter*	.168	4.11	.968	3 - 12
8121	*Aeromonas*	.157	4.41	.996	6 - 24
8122	*Acinetobacter*	.363	1.91	.991	3 - 9
8123	*Aeromonas*	.365	1.90	.993	3 - 12
8124	*Vibrio*	.136	5.08	.934	3 - 15
8125	*Alcaligenes* 1	.238	2.91	.974	3 - 12
8126	*Alcaligenes* 2	.185	3.74	.923	3 - 15
	Pseudomonas marina	.411	1.69	.999	3 - 9

Table II.8a

Effects of Supplemental Nitrogen on Bacterial Biomass and Dry Weight Conversion Ratio of Five Bacterial Isolates Cultured on BCSF

isolate	nitrogen supplement (mg NH_4SO_4/l) (mean ± SD)	bacterial biomass (mg/l)	dry weight conversion ratio (mg/g BCSF)
Pseudomonas marina	0	45 ± 10	90
	38	63 ± 7.3	127
	76	58 ± 10	116
	152	50 ± 12	101
Pseudomonas sp.	0	27 ± 5.5	53.6
	38	34 ± 6.6	68.9
	76	35 ± 4.9	70.9
	152	31 ± 7.4	61.6
Micrococcus sp.	0	24 ± 2.5	47.4
	38	25 ± 4.0	50.2
	76	26 ± 4.2	51.5
	152	21 ± 3.5	41.4
Aeromonas sp.	0	31 ± 8.7	62.2
	38	44 ± 7.9	88.3
	76	40 ± 5.5	80.9
	152	42 ± 9.4	83.6
Flavobacter sp.	0	39 ± 4.0	78.9
	38	47 ± 5.5	95.0
	76	54 ± 8.9	108
	152	41 ± 5.6	81.6

P. marina (VIMS stock isolate), and Aeromonas sp. (8123). Two other isolates were chosen because they had characteristics common to many other genera of marine bacteria, which might influence their utility for mariculture. Micrococcus sp. (5013) was examined because it is a gram positive coccus and Flavobacter sp. because it is a pigmented form.

Bacterial biomass, measured as dry weight, was determined following supplementation with 0, 38, 76 and 152 mg/l of ammonium sulfate (Table II.8a). Two-way factorial analysis of variance (Table II.8b) indicated that both bacterial species and ammonium sulfate supplement significantly affected bacterial dry weight ($p<.01$). There was no significant interaction ($p<.05$) between bacterial species and ammonium sulfate supplements on biomass. Largest bacterial biomass, averaged over all nitrogen levels, was obtained with Pseudomonas marina (54 mg/l) followed by Flavobacter sp. (45.2 mg/l), Aeromonas sp. (39.2 mg/l), Pseudomonas sp. (31.8/l), and Micrococcus sp. (24.2 mg/l). Results of the Student-Newman-Keuls multiple range test (Zar, 1984) indicated that Flavobacter sp. and Aeromonas sp. were not significantly different ($p=0.05$) from each other in regard to biomass but all other species were significantly different. P. marina had the largest biomass at all levels of ammonium sulfate and Micrococcus sp. always had the smallest. Flavobacter sp. had the second largest biomass at three of the four ammonium sulfate levels.

The greatest bacterial biomass when averaged over all bacterial species was obtained with 38 mg/l of ammonium sulfate (42.8 mg/l dry weight), followed by 76 mg/l (42.4 mg/l dry weight), 152 mg/l (36.8 mg/l dry weight), and 0 mg/l (33.1 mg/l dry weight). Multiple comparisons performed by the Student-Newman-Keuls test indicated that the 0 mg/l

Table II.8b

Two-way ANOVA Comparing the Effects of Supplemental Nitrogen and Bacterial Isolate on the Bacterial Biomass

Source of variance	Sum of Squares	d.f.	Mean square	F ratio	P
bacterial isolate	6,619.1	4	1654.7	31.99	<.001
nitrogen supplement	990.7	3	330.2	6.38	<.001
interaction	464.5	12	38.7	.74	.697
residual	2,068.6	40	51.7	--	--
total	10,142.9	59	171.9		

Student-Newman-Keuls test for significant differences among bacterial isolates. Bars underline equal means (alpha =0.05)

P. marina > *Flavobacter sp.* > *Pseudomonas sp.* > *Pseudomonas sp.* > *Micrococcus sp.*

Student-Newman-Keuls test for significant differences among nitrogen levels. Bars underline equal means (alpha = 0.05)

38 mg/l > 76 mg/l > 152 mg/l > 0 mg/l

level of ammonium sulfate was significantly different (p=.05) from the other three levels of ammonium sulfate but that no significant differences existed among the other three levels. Bacterial concentrations were lowest for four of the five species when no ammonium sulfate was added. Although not statistically significant, bacterial biomass decreased at the highest level of ammonium sulfate. In four of the five species, the bacterial biomass at 152 mg/l ammonium sulfate was less than the biomass at 38 and 76 mg/l. Micrococcus sp., however, had its lowest overall concentration with 152 mg/l of ammonium sulfate and its second lowest dry weight when no ammonium sulfate was added.

Carbon conversion ratios for the five bacterial species (Table II.9) showed a trend similar to that for bacterial dry weights. P. marina, again had the highest carbon conversion ratio, followed by Flavobacter sp., Aeromonas sp., Pseudomonas sp., and Micrococcus sp. Single factor analysis of variance indicated that bacterial species had a significant effect upon carbon conversion ratios ($p<.01$). Multiple comparisons performed by the Student-Newman-Keuls test ($p=.05$) indicated that P. marina was significantly different from all other species and that Flavobacter sp. was significantly different from Micrococcus sp. No significant differences existed among Aeromonas sp., Pseudomonas sp., and Micrococcus sp. Bacterial species had a small, but still significant ($p=.014$) effect upon yield. The Student-Newman-Keuls test revealed significant differences ($p=.05$) only between the highest (P. marina) and lowest (Micrococcus sp.) yields. The rank order of the intermediate species was different from that based on carbon conversion ratios.

Table II.9

Carbon Conversion Ratios and Yields of Five Bacterial Isolates Cultured on BCSF Without Supplemental Nitrogen

isolate	carbon conversion efficiency (mg C_{cells}/g C_{BCSF})	yield (percentage)
Pseudomonas marina	146 ± 18.7	27.4 ± 3.5
Pseudomonas sp.	80.4 ± 1.7	23.7 ± 3.2
Micrococcus sp.	67.5 ± 7.9	16.8 ± 3.6
Aeromonas sp.	102 ± 22.5	22.7 ± 2.6
Flavobacter sp.	113 ± 10.6	20.5 ± 1.5

One-way ANOVA of the effect of bacterial isolate upon carbon conversion ratio

Source of variation	Sum of squares	d.f.	Mean square	F ratio	P
between treatments	438.0	4	109.5	10.439	.0014
within treatments	104.9	10	10.4		

Student-Newman-Keuls test for significant differences among bacterial isolates. Bars underline equal means (alpha = 0.05)

P. marina > *Flavobacter* sp. > *Aeromonas* sp. > *Pseudomonas* sp. > *Micrococcus* sp.

One-way ANOVA of the effect of bacterial isolate upon yield

Source of variation	Sum of squares	d.f.	Mean square	F ratio	P
between treatments	.0185	4	.0046	5.347	.0145
within treatments	.0087	10	.0009		

Student-Newman-Keuls test for significant differences among bacterial isolates. Bars underline equal means (alpha = 0.05)

P. marina > *Pseudomonas* sp. > *Aeromonas* sp. > *Flavobacter* sp. > *Micrococcus* sp.

Supplemental nitrogen had a significant effect ($p<.05$) upon the carbon conversion ratio of P. marina similar to its effect upon bacterial dry weight (Table II.10), but the effect was less pronounced for carbon conversion ratios. The greatest carbon conversion ratio occurred with 38 mg/l of ammonium sulfate followed by 76 mg/l, 152 mg/l, and 0 mg/l. Treatment effects, however, could be distinguished only between 38 mg/l and 0 mg/l, using the Student-Newman-Keuls test. In the case of dry weight, all amounts of nitrogen addition were significantly different from no nitrogen addition. Analysis of variance indicated no significant differences among the yields of P. marina at any level of nitrogen. Yields ranged from 27.4% for no nitrogen addition to 38.5% with 38 mg/l.

Table II.10

Effect of Supplemental Nitrogen on Carbon Conversion Ratios and Yield of *Pseudomonas marina*

nitrogen supplement (mg NH_4SO_4)	carbon conversion ratio (mg C_{cell}/g C_{SBCS})	yield (percentage)
0	146 + 18.7	27.4 + 3.5
38	203 + 20.7	38.5 + 2.5
76	199 + 28.2	35.8 + 5.6
152	158 + 20.8	31.2 + 4.7

One-way ANOVA of the effect of supplemental nitrogen upon carbon conversion ratio

Source of variation	Sum of Squares	d.f.	Mean square	F ratio	P
between treatments	257.6	3	85.8	4.977	.0309
within treatments	138.0	8	17.2		

Student-Newman-Keuls test for significance among nitrogen levels. Bars underline equal means.

38 mg/l > 76 mg/l > 152 mg/l > 0 mg/l

One-way ANOVA of the effect of supplemental nitrogen upon yield

Source of variation	Sum of Squares	d.f.	Mean square	F ratio	P
between treatments	0.0220	3	0.0073	4.004	.051*
within treatments	0.0146	8	0.0018		

* not significant at alpha = 0.05

DISCUSSION

As the name "brewers condensed solubles" (BCS) suggests, the nutrients in BCS were primarily dissolved (94.7% soluble solids). Since no attempt was made to correct for soluble solids remaining in the packed centrifuge pellet, 94.7% is an underestimate. The liquid fraction of BCS is primarily water but may contain small amounts of ethanol since spilled beer is part of the waste stream used to make BCS. In an analysis of twelve BCS samples from the Anheuser Busch brewery in Merrimack, MA, Sebree et al. (1982) found that soluble solids accounted for 93% of the total solids. Using a high drying temperature (100°C), they found the total solids content to be 44%. A low drying temperature (80°C) was chosen in this study to minimize loss of volatile solids and to prevent charring.

Sebree et al. (1982) found that BCS contained on a dry weight basis (db), 75% carbohydrate (by the phenol sulfuric acid method) and 9% (db) protein (calculated from the Kjeldahl nitrogen value). In this study, the carbohydrate content was 72.8% (db) and the protein content was 8.6% (db). However, suspended solids, which accounted for only 5.3% of the dry weight, were found to contain 42.1% of the total protein in the BCS. Suspended solids are therefore 35% (db) protein. BCSF had a correspondingly small protein content of 5.3% (db).

The carbon content of the carbohydrate and protein averaged only 80% of the measured TOC. The TOC (42.8 mg/g) not accounted for may

represent in part other organic compounds such as ethanol, lipids, and complex polysaccharides. In their analysis of BCS, Sebree et al. (1982) found that BCS contained 1.4% (db) fat and 2% (db) crude fibre. These together, if entirely in the BCSF fraction would be unlikely to contribute together more than 11 mg/g of TOC, based on the percentage of carbon in lipid (75%) and fibre (40%). Sebree et al. (1982) reported the following percentages of the various carbohydrate components (total dry weight): dextrose 6%, maltose 32%, maltotriose 32%, and large maltodextrins 27%. Large maltodextrins may be significantly underestimated by the anthrone method (Herbert et al. 1971) but would still be quantified by the TOC analyzer.

The discrepancy between the measured TOC and estimated organic carbon is reflected in the C:N ratios. C:N ratios calculated from the TOC measurements averaged 25% greater than those calculated from the estimated percentage carbon in carbohydrate and protein. In this study, TOC:N ratios were used to calculate the amount of supplementary nitrogen required for maximal biomass production from BCSF. These were believed to more accurately reflect the relationship of carbon and nitrogen than calculated values based on carbohydrates and protein.

Industrial by-products similar to BCS which have been used extensively as nutrient sources for microbial biomass production include beet, corn, and wood molasses. The composition of these by-products has been summarized by Riviere (1975). Beet molasses has a higher solids content (85.5 wt %) than BCS. Total carbohydrates, however, are a smaller fraction of the total solids (52% db) and nitrogenous compounds are a larger portion (13% db) than in BCS. Carbohydrates in beet molasses are almost entirely in the form of sucrose. Cane molasses contains 62% sugar (32% sucrose, 14% glucose, and 16% fructose) but less

organic nitrogen than beet molasses. Wood molasses, a by-product of the pulp paper industry, contains 50 to 62 wt % solids and is 48.5 to 51.5% (wt %) carbohydrates. Hexoses comprise 65 to 85% of the carbohydrates; the remainder are pentoses. The nitrogen content of wood molasses is only 0.065 wt %, which is considerably lower than that of BCS. BCS is similar to other organic byproducts in total solids, carbohydrate, and protein content. It differs mainly in its carbohydrate composition which is mainly maltose, maltotriose, and large maltodextrins (Sabree et al, 1982).

The nitrogen content of BCSF is less and the C:N ratio is greater than those found in wastes of other food and beverage processing industries. BCSF has a protein content of 27.8 mg/g or 5.3% db and its TOC:N ratio is 42.6. Tomlinson (1976) reported organic carbon:nitrogen ratios for other food industry wastes used in a study of single cell protein (SCP) OOoduction: brewery, 30.6; distillery, 13.0; beet sugar processing, 16.6; canning, 34.6; and potato processing, 14.1. Brewery wastes in general are deficient in nitrogen. Because of their correspondingly high C:N ratios they have been used as carbon sources for the denitrification of domestic effluents (Wilson and Newton, 1973). Cellulosic agricultural wastes with much higher C:N ratios than BCS, have been used successfully for SCP production after the addition of supplemental nitrogen (Rockwell, 1976). Wheat straw is 0.3-0.5% N with a C:N ratio of 128-150, oat straw has a N content of 1.1% and a C:N ratio of 48, and sawdust is 0.1% N with a C:N ratio of 200-500 (Golueke, 1977).

Since the objective of this study required the isolation of strains of bacteria capable of growth on BCSF, enrichment cultures were

employed with BCSF as the sole added carbon source. If these bacteria are to be used for estuarine mariculture, they must also be capable of growth under estuarine conditions. York River water, therefore, was used as an inoculum and the salinity of the cultures was kept at 20°/oo. BCSF substrate concentration was 1.0 g/l, which is considerably lower than the 20 to 40 g/l typically used in enrichment media for the selection of chemoorganotrophs (Veldkamp, 1970). At this lower substrate concentration, the natural buffering capacity of York River water was sufficient to maintain pH in the range of 6.7 to 7.8. Higher substrate concentrations would require substantial buffering to neutralize the resulting high levels of organic acids.

Batch cultures select for opportunistic species adapted for growth at high nutrient concentrations and with low substrate specificity (Jannasch, 1967). These characteristics, however are desirable for a wastewater mariculture system, where the objective is to maximize production of bacterial biomass.

The initial substrate concentration in batch cultures, which is in great excess, selects for strains with a high maximum specific growth rate (Jannasch, 1967). In the pelagic marine environment, which is considered nutrient limited, bacteria most important in nutrient cycling may be those which are able to utilize low substrate concentrations and which have low maximum growth rates. Most marine bacteria have growth characteristics unsuitable for waste water mariculture. These enrichment cultures selected for species with high maximum specific growth rates. The isolates obtained do not have growth and uptake rates representative of typical marine bacterial flora.

Batch cultures also select for auxotrophs (Parkes, 1982). Growth factors are released into the cultural medium by lysis of cells in the original inoculum and in the early stages of the growth curve. Bacteria which are favored by these growth factors may dominate the culture. These bacterial strains will not grow well when inoculated into similar media lacking the added growth factors. BCSF is a rich source of vitamins and growth factors even without the addition of cell lysis by-products. Its vitamin content is similar to that of malt extract which is used to supplement enrichment cultures (Bridson and Brecker, 1970). The isolates obtained from enrichment culture were not tested for auxotrophic requirements, but if these do exist, their needs should be adequately met by BCSF.

Identified bacterial isolates all belonged to genera exhibiting chemoorganotrophic nutrition and are capable of growth on a wide variety of organic compounds. All isolates were capable of growth on maltose and sucrose, the major consituents of BCSF. The ability to utilize maltose and sucrose is widespread among the isolated genera. The enrichment cultures were all strongly aerated, so it is not surprising that most isolates utilized these carbohydrates by oxidative respiration. Ten isolates belonged to genera with strictly aerobic metabolism and the remaining four isolates are facultative anaerobes.

The six genera of gram negative bacteria isolated on BCS medium are all common in the Cheaspeake Bay and adjacent coastal waters. In a bacteriological study of the upper Chesapeake Bay, Lovelace et al. (1965) found 56% of the isolates to be Vibrio, 18% to be Pseudomonas, and 6% to be Flavobacter. Cook and Goldman (1976) made 54 isolates from the upper Chesapeake Bay and found 37% to be Vibrio, 15% to be

Alcaligenes, 7.4% to be Acinetobacter, and 5.6% to be Pseudomonas. Austin et al. (1979) sampled the lower Chesapeake Bay off Cape Charles and also found Vibrio to be numerically dominant; out of 80 isolates, 34 were Vibrio. Pseudomonas (11 isolates) and Acinetobacter (3 isolates) were also dominant taxa. In a study of the Middle Atlantic continental shelf, Kator (1977) sampled the surface microlayer, surface water, bottom water, and sediments during four seasons. Pseudomonas dominated in the surface waters during all seasons with Flavobacter the second most frequent taxon. The third most frequent genus was Alcaligenes or Vibrio depending on the season. When the entire water column and sediments were considered, Flavobacter was the most frequently isolated genus and Pseudomonas or Alcaligenes was secondary in total number of isolates. The sixth most prevalent genus was Aeromonas which has not been reported as commonly as the other genera although it was isolated by Cook and Goldman (1976) and Austin et al. (1979).

Only 2 of 15 isolates were gram positive; gram positive bacteria usually represent less than 15% of isolates from the water column of estuarine and coastal waters. Simidu and Aiso (1962) examined 475 isolates from Kamogawa Bay and found that 6% were gram-positive. Sieburth (1967) found the average occurrence of gram-positive isolates in mid-Narragansett Bay to be 2.6%. Murchelano and Brown (1970) found only 0.5% of their 649 isolates from Long Island Sound to be gram positive.

A wide range of specific growth rates were exhibited by bacteria isolated in this study. Most of the bacteria did not have exceptionally high growth rates. Selection for many other factors besides high specific growth rates may have occurred in the enrichment cultures.

Predation avoidance mechanisms, ability to colonize vessel walls, and ability to withstand high concentrations of cell lysis by-products are other possibilities. It is especially difficult to predict selection pressures when the substrate is a complex mixture such as BCSF. Although all isolates were shown to have the ability to grow on glucose, maltose, and sucrose, their relative abilities to degrade and grow on more complex polysacharides may be important in determining their competitive abilities. Some isolates may also have been selected because they are able to grow on metabolic by-products of other species.

Fast growing isolates ($u>0.3\ h^{-1}$) had specific growth rates similar to maximum values reported for bacteria grown aerobically on easily degraded substrates. Since high specific growth rates occur on easily degraded substrates (Grady and Lim, 1980), at least some components of the BCSF must be easily degraded. Maltose and sucrose are obvious examples. Subatances toxic to bacteria must likewise be absent from BCSF . Growth rates of 0.3 to $0.4\ h^{-1}$ are close to the maximum for many bacterial species when grown at $20^{o}C$ on a wide variety of easily assimilated substrates (Mohr and Krawiec, 1980; Ratkowsky et al., 1983). Maximum specific growth rates for mixed cultures of bacteria grown with glucose as the sole carbon source are typically in the range of 0.3 to $0.4\ h^{-1}$ (Piel and Gaudy, 1970; Muck and Grady, 1974). Growth rates on lactose range from 0.2 to $0.53\ h^{-1}$, on sucrose from 0.28 to $0.55\ h^{-1}$, and on acetic acid from 0.29 to $0.36\ h^{-1}$ (Piel and Gaudy, 1971). Pure cultures of bacteria from sewage, when cultured in a rich nutrient broth supplemented with sucrose at $25^{o}C$, had specific growth rates of 0.597 h^{-1}(*Enterobacter* sp.), 0.368 h^{-1} (*Alcaligenes* sp.), 0.333

h^{-1}(*Pseudomonas* sp.) and 0.252 h^{-1} (*Brevibacterium* sp.) (Lester et al., 1979). Bacterial growth rate is highly dependent on temperature and many high growth rates reported were measured at higher temperatures than used in this study.

Most single cell biomass production studies with wastes similar to BCSF have involved fungi rather than bacteria. Specific growth rates for fungi are often less than that reported for the fastest growing bacteria. However, in studies in which environmental factors for fungal growth were optimized (temperature, nutrient concentration, oxygen level and pH), specific growth rates were comparable to those for bacteria. *Aspergillus niger* batch cultured on brewery spent grain liquore, the major component of BCSF, had a specific growth rate of 0.016 h^{-1}(Hang et al., 1975). *Geotrichum candidum*, *Candida krusei*, and *Hansenula anomala* cultured together exhibited growth rates of 0.14 h^{-1} when batch cultured on whiskey distillery spent wash, a waste with a higher protein content than BCSF (Barker, Quinn, and Marchant, 1982). In continuous culture, *H. anomala* and *C. krusei* maintained themselves at dilution rates up to 0.35 h^{-1}, while *G. candidum* washed out at dilution rates greater than 0.20 h^{-1}. Quinn and Marchant (1980) reported the maximum biomass production (0.125 h^{-1}) of *G. candidum* on distillery spent wash to occur at 22°C. Sheehan and Greenfield (1980) reviewed reports on biomass production from distillery wastewaters. Typical dilution rates reported in this review were 0.166 h^{-1} for *Candida utilis* and 0.083 h^{-1} for *Candida tropicalis*. Cabib et al. (1983) cultured *C. utilis* on stillage from cane molasses alcohol and reported growth rates of 0.19 to 0.24 h^{-1} in batch culture. Continuous cultures achieved steady states at dilution rates up to 0.27 h^{-1}. Reed and Peppler (1973) reported

specific growth rates of Saccharomyces carevisiae on cane molasses in continuous culture to range from 0.20 to 0.25 h^{-1}.

The greatest bacterial biomass was measured with P. marina when the BCSF medium was supplemented with 38 mg/l of ammonium sulfate. On a dry weight basis 127 mg of cells were produced per gram of BCSF and when measured as organic carbon 203 mg per gram of carbon in BCSF. The cells are estimated to be 63% carbon, which is greater than the often cited theoretical carbon content of 53.1% based on a formula weight of $C_5H_7O_2N$ (Grady and Lim, 1980). The cellular carbon content may be slightly overestimated due to loss of cells and leakage of cellular constituents during the centrifugation and washing steps of the dry weight determinations. These losses would not be associated with TOC estimates of cell biomass. High carbon content may also have resulted from growth on a rich carbon medium. Excess carbon and energy sources may lead to production of energy reserves which constitute a high proportion of biomass dry weight (Pert, 1975). Cells grown on brewery wastes have previously been reported to contain high percentages of polysaccharide storage products (Takii, 1977).

The dry weight conversion ratio of BCSF by P. marina is similar to that of heterotrophic bacteria on nutrient broth. Escherichia coli cultured on nutrient broth at a concentration of 8 g/l will produce 1 g/l of cells, which is a conversion ratio of 125 mg/g. Considerably higher conversion ratios may occur (375 mg/g) for a medium rich in easily assimilated substrates (Riviere, 1977). Conversion ratios in this study may have been reduced by loss from cells of soluble organic compounds. These compounds may be shunted metabolic intermediates or may represent an inadvertent leakage of cell constituents (Dennis and

Irvine, 1981). An additional loss of fixed carbon in this study may have been the loss of bacterial exopolymers (e.g. slime) during centrifugation.

Bacterial biomass differed among the five species when measured as dry weight or organic carbon, although the differences were less when measured as organic carbon. Yields of the five species differed much less than concentrations and yields of only two species differed significantly. Bacterial yields for aerobic growth on a given substrate are usually very similar for all species (Payne, 1970). Differences in bacterial biomass may be due partially to differences in yields of the species; P. marina had both the highest yield and the greatest biomass while Micrococcus sp. had the lowest yield and the lowest biomass.

Various factors may influence the final bacterial biomass. All components of BCSF may not be equally available to all species; those bacteria able to metabolize a wider range of components would produce more biomass. Optimal environmental conditions (e.g. temperature, salinty, pH, aeration, specific ionic requirements) for synthesizing biomass may be different for these species. The environmental conditions employed may have been more favorable for those species with the higher biomass concentrations.

Addition of nitrogen supplements had a significant effect upon bacterial conversion ratios measured as either dry weight or organic carbon. This suggests that the concentration of nitrogen in BCSF may be limiting to bacterial growth. Addition of 78 mg of ammonium sulfate per g of BCSF was calculated to lower the BCSF C:N ratio from 53.5:1 to 10:1. The optimal C:N ratio for a given culture medium is a function of the stoichiometry of bacterial growth (Grady and Lim, 1980). A C:N

ratio of 10:1 is widely used in production of SCP (Litchfield, 1979), but values up to 20:1 have also been suggested as optimal (Gray et al., 1980). The 10:1 ratio is based on the assumptions that a bacterial cell is 50% carbon and 10% nitrogen and that the growth yield is 50%. No significant differences in bacterial conversion existed between any levels of ammonium sulfate addition. Addition of 38 mg/g of BCSF should have resulted in less biomass than addition of 76 mg/g if 76 mg/g produced the optimal C:N ratio. A similar result was obtained by Tomlinson (1976) with Candida utilis cultured on brewery wastes supplemented with ammonium sulfate. Biomass conversion was similar at C:N ratios of 15 and 30.

The optimal C:N ratio for BCSF bioconversion appears to be greater than 10:1. This would be expected since observed yields were considerably lower than 50% and the carbon content of the cells exceeded 50%. addition, if some carbon constituents of the BCSF were not available to the bacteria, the amount of nitrogen required would be correspondingly less. The C:N ratio of BCS would be effectively lowered.

The biomass conversion ratios decreased slightly with supplements of ammonium sulfate greater than 38 mg/g of BCSF. It is not known why this occurred. A similar result was noted by Shannon and Stevenson (1975), who reported that the biomass of Candida gigantea cultured on brewery grain press liquor was maximal when supplemented with 0.05% nitrogen but decreased with 0.10% and 0.15% nitrogen. The same pattern occurred when brewery trub press liquor was used as a nutrient source.

Wastes similar in composition to BCS have also been found to require addition of supplemental nitrogen in order to maximize biomass

conversion. Shannon and Stevenson (1975) found that conversion of brewery wastes to yeast biomass could in some cases be more than doubled by addition of nitrogen in the form of ammonium sulfate. Vriens et al. (1983) similarly found that yields of microbial biomass from brewery wastes were significantly greater when nitrogen was added. Rische (1966) found that beet molasses stillage supplemented with ammonium sulfate led to a 25% increase in the biomass yield of C. utilis. Quinn and Marchant (1978), however, culturing C. candidum on distillery wastes did not find consistent increases in biomass following supplementation with nitrogen.

Although bacterial conversion ratios were low for the five species examined, yields were high. Yields exceeded 30% for P. marina at all levels of nitrogen supplementation and the highest yield was 38.5% with 38 mg of ammonium sulfate. These yields, however, may be inflated if significant amounts of fixed carbon are being lost from the cells. The TOC analysis did not differentiate soluble organic carbon released into the medium from that in the original BCSF medium. A given amount of biomass would appear to be synthesized from less medium than was actually consumed.

Bell (1972) reviewed a large number of studies on bacterial yield expressed as g dry weight per g of substrate carbon. Aerobacter aerogenes cultured on maltose had a yield of 1.03 and the mean yield was 1.00 for several microorganisms cultured on sucrose, and 1.11 for microorganisms on glucose. Mean yields for aerobic growth on simple sugars was 1.07 which is equivalent to 0.56 g carbon per g substrate carbon, if a theoretical carbon value for bacteria of 52% is employed. The maximum yield in this study was 0.385, which is 69% of the mean

value. The mean value of 0.56 is based on studies in which conditions for most efficient substrate utilization had been optimized. Growth under less than optimal conditions leads to increased maintenance requirements and reduced yields (Harrison, 1978). If yields could be raised to the mean value by optimizing the environmental conditions, a 31% increase in biomass would result. The studies, however, on which this mean was based employed single substrates of mono- or disaccharides. Yields may be lower if based on mixtures of substrates with more complex components.

Biomass conversion ratios might also be increased by using mixed cultures of microorganisms. The "spent" BCSF medium contains a mixture of unused BCSF components and secreted metabolic intermediates. A mixture of bacteria would have a greater range of hydrolytic enzymes and be more likely to metabolize a wider range of BCSF components than any single species. Secreted or leaked metabolic intermediates are readily metabolized by other species in a mixed culture (Obayashi and Gaudy, 1973). Mixed cultures have many advantages over monocultures, especially when the culture medium is a heterogeneous mixture such as BCSF. Treatment of domestic sewage, a very heterogeneous mixture of substrates, can only be achieved by a mixed culture (La Riviere, 1977). Mixed cultures have been highly successful for production of SCP from natural gas, a mixture of substrates analagous to BCS. Harrison (1978) described the use of careful recombinations of species, termed "structured mixed cultures" as opposed to crude enrichments, for SCP production. Besides increases in biomass conversions and yields, other advantages of mixed cultures are increased stability, resistance to

contamination, lowered susceptibility to foaming, and reduced vitamin requirements.

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SECTION III

Growth of Colorless Flagellates

INTRODUCTION

Colorless flagellates have not previously been considered as potential food organisms for use in mariculture because their importance in marine food chains was not recognized until recently. Standard bacteriological methods were not adequate for their enumeration and classical techniques of plankton ecology either overlooked them or did not differentiate them from phytoplankton. Not until membrane filters and fluorescence microscopy became available for use in marine ecology was there a simple way to quantify colorless flagellates. Colorless flagellates are now considered to be important intermediate links between bacteria and large zooplankton in the trophodynamics of marine ecosystems (Kopylov et al., 1981; Azam et al., 1983). They may also be an additional link between small phytoplankton and zooplankton (Goldman et al., 1985). Little is still known about the grazing of colorless flagellates by macrobenthic organisms.

In nature, flagellates are adapted to exploiting a food source which is spatially and temporally heterogeneous (Fenchel, 1982c). Studies in natural ecosystems (Yongue and Cairns, 1971) and in microcosms (Henebry and Cairns, 1980) have demonstrated flagellates to be the dominant species among the protozoa in the early development of fouling communities. They may be particularly abundant in eutrophic

environments (Henebry and Cairns, 1980). Highly polluted environments often have large populations of flagellates, which are not as sensitive to pollution as are their large predators (Sorokin, 1981). Flagellates have similarly been demonstrated to be the early colonizing species in the development of activated sludge (Curds, 1966), a waste treatment system which is similar to the wastewater mariculture system being developed in this study.

Colorless flagellates are good candidates for use in mariculture because they have r-selected strategies for survival in the natural environment. r-Selected organisms are successful pioneer species and typically have high reproductive rates, density-independent mortality, and the ability to use various resources (Pianka, 1970). When inoculated into the ecological vacuum of a mariculture system, an artificial environment with initially unlimited nutrients and no competitors, they are able to realize their high intrinsic productivity. Many flagellate species are considered to be "weed" organisms by mariculturists and are major nuisances in algal culture facilities where they contaminate and outgrow algal monocultures (Lucas, 1968; M. Roberts, Virginia Institute of Marine Science, personal communication). Their ability to use variable resources is an asset in a mariculture system in which the nutrient composition may vary, such as in this study.

In the few studies in which colorless flagellates have been cultured, no attempts were made to find their optimal environmental conditions for growth. Most studies were concerned with maintaining cultures for taxonomic purposes (Gold et al., 1970; Leadbeater and Morton, 1974) or for physiological investigations (Lee, 1978; Nultsch

and Hader, 1984). More recently, studies have cultured flagellates to elucidate their role in marine ecosystems (Fenchel, 1982a; Sherr et al., 1983; Davis and Sieburth, 1984). No published studies exist on culturing flagellates with the aim of using them as food organisms in mariculture.

The effects of several environmental parameters on the growth of colorless flagellates were investigated in this study in order to determine both the optimal conditions for growth an the sensitivity of the flagellates to environmental changes. Such information is essential if flagellates are to be used in a mariculture system. Salinity, temperature, agitation and bacterial concentration were among the environmental parameters examined. Flagellate growth on dissolved nutrients was compared to growth on a bacterial diet and the suitability of various bacterial isolates was examined. In addition, yields of flagellates cultured on bacteria were determined. The experiments were designed with the overall objective of obtaining information to be used in establishing a mariculture operation rather than being purely an investigation of protozoan physiology. For this reason, the experiments were carried out in batch cultures, which is how most microorganisms are raised for food.

MATERIALS AND METHODS

Eighteen isolates of colorless flagellates were screened of which five species were selected for use in this study. They included two species of chrysomonads, two species of bodonids, and a choanoflagellate. Only one chrysomonad, Paraphysomonas vestita, has been identified to the species level. The other unidentified species were designated as chrysomonad 1, bodonid 1, bodonid 2, and choanoflagellate 1. All of these organisms were isolated from the York River at Gloucester Point, VA by members of the Virginia Institute of Marine Science (choanoflagellate 1 by S. Arnold, P. vestita, bodonid 1 and chrysomonad 1 by L. Haas, and bodonid 2 by D. Guasman). These five were chosen because they were relatively easy to culture on a bacterial diet, had faster growth rates than other isolates, and represented different taxonomic groups.

Linear dimensions of living specimens were measured from an exponentially growing culture by phase microscopy at 1,000X magnification. Twenty cells were measured of each species. Volumes were calculated from measurements of either their diameter or width and length using the formula of either a sphere ($V=4/3\ r^3$; where r=radius) or a rotation ellipsoid ($V=4/3\ ab^2$; where a=length/2, b=width/2).

The direct growth of colorless flagellates on Brewers Condensed Solubles Filtrate (BCSF) was compared with growth of flagellates fed bacteria raised on BCSF. For each species of flagellate four sets of

triplicate 500 ml flasks were filled with 250 ml of sterile, filtered (0.2 u) York River water to which was added 0.25 g of BCSF. BCSF was prepared as described in Section II. Four ml of an antibiotic solution containing 4.0 mg/ml of sodium penicillin G (1675 U/mg) and 4.0 mg/ml of streptomycin sulfate were added to the first set of flasks to prevent growth of bacteria. All culture media were inoculated with 10^7 cells of Pseudomonas marina from an exponential phase culture and plugged with cotton stoppers. The flasks were kept on a rotary shaker table and maintained at 22°C. After 24 hours the bacteria in the second, third, and fourth sets of flasks had reached stationary phase while no growth had occurred in the first set. The bacteria in the third set of flasks were harvested by centrifugation at 10,000 g for 20 minutes. The centrifugate was discarded and the cells were resuspended in 250 ml of sterile York river water. Four ml of the antibiotic solution were added to the resuspended bacteria in the fourth set of flasks. All twelve flasks were inoculated with 5×10^4 cells of the given species of flagellate. Forty eight hours after inoculation, flagellates in the flasks were sampled and enumerated.

The effects of shaking, salinity, temperature, and bacterial concentration on growth of the five flagellate species were individually examined in a series of four experiments. The experiments were performed in triplicate 500 ml flasks containing filtered (0.2 um) sterile York River water enriched with BCSF and inoculated with P. marina as previously described but without addition of any antibiotics. The flasks were placed on a rotary shaker at 100 rpm and maintained at 20°C. The bacteria were cultured for 24 hours at which time their population was in stationary phase and the flasks were inoculated with

flagellates. The bacterial concentration experiment differed slightly in its initial protocol as described below. The flagellate inoculum was taken from a recently transferred stock culture that was carefully monitored to ensure that the cells were in exponential growth. The time at which the flagellates were added was considered to be time zero. Five ml samples were taken from each flask at 0, 24, 48, 72, and 96 hours and fixed with 0.5 ml of an acidified formalin preservative. The preservative consisted of a 15% solution of formalin (v/v) mixed with a 2.0% solution of acetic acid (v/v) in a ratio of 5:2. The samples were kept refrigerated at 7°C until processing which occurred within one week.

Preserved samples were stained with 100 ul of either eosin Y (0.2 g/100 ml) or aniline blue (0.2 g/100 ml). Although both dyes were satisfactory for staining flagellates for enumeration, aniline blue was used for the majority of samples because with it the flagella of stained cells were pronounced. Aniline blue is colored only under acidic conditions and samples which were highly diluted after fixing had to be acidified with acetic acid to prevent loss of color. Stained samples were vacumn filtered onto 25 mm diameter Gelman GA-6 cellulose triacetate membrane filters (0.45 um). The filters were dried at 40°C on glass slides and cleared with immersion oil (Cargille, type B). Flagellates were enumerated at a magnification of either 200 X or 430 X depending on size, shape, and intensity of staining. In general, only P. vestita and bodonid 1 could be enumerated at the lower magnification. Forty fields or 200 cells were counted on each slide and two slides were prepared from each sample. Cell concentrations exceeding 10^5 cells/ml were counted in a hemacytometer cell (A. O. Spencer "Brightline")

according to the procedure of Guillard (1973). Two counts were made of each sample.

Specific growth rates (k) for each treatment were calculated from the increase in flagellate concentrations during the exponential phase of growth. The linear relationship between the natural logarithm of cell concentration (y) and sampling time (x) was determined by a least squares regression model. The slope of this line, in units of hours $^{-1}$, was considered to be the specific growth rate. The least squares calculations were performed on a Prime computer (model 850) using an analysis of covariance (ANCOVA) program developed by the National Marine Fisheries Service (Russo, 1977). The ANCOVA program also compared the specific growth rates (slopes) for each treatment and determined if they were significantly different at the five percent significance level. The Student-Newman-Keuls multiple range test was used to determine which specific growth rates differed significantly at a five percent significance level (Zar, 1974). The specific growth rates were converted to divisions/day by multiplying by 24 hours and dividing by the natural logarithm of 2.

In the shaking experiment the rotary tables were set at 0, 100, 150, and 200 rpm while in the other three experiments they were kept at 100 rpm. Dissolved oxygen concentrations were measured initially in the shaking experiment and at the 24 hour sampling period using a YSI model 57 oxygen meter (YSI corp., Yellow Springs, OH.). In the salinity experiment, salinities of 5, 15, 25, and 35 ^{o}/oo were tested but in the other experiments the salinity was maintained at 17 ^{o}/oo. Stock cultures of flagellates were maintained at 17 ^{o}/oo and were transferred directly into different salinities without any attempt at acclimation.

All experiments were performed at 20°C except for the temperature experiment in which temperatures of 5, 13, 21, 26, and 31°C were tested. Stock cultures were maintained at 20°C and were transferred directly into flasks at the different temperatures without any temperature acclimation. In all but the bacterial concentration experiment, each flask was enriched with 0.25 g of BCSF which produced approximately 10^8 cells/ml of P. marina. The initial protocol for the bacterial concentration experiment differed in that the bacteria were first cultured in an 18 l carboy containing 5.0 g/l of BCSF. When this culture reached stationary phase, it was diluted with sterile York River water to the appropriate bacterial concentration and dispensed into 500 ml flasks. Bacterial concentrations of 10^7, 5.0×10^7, 10^8, and 5×10^8 cells/ml were tested.

Yields of each flagellate species were determined from the stationary phase cultures in the bacterial concentration experiment. The biomass of flagellates was determined by filtering the culture through glass fiber filters (Whatman GF/F, 24 mm diameter). Each filter was previously rinsed with 100 ml of particle free distilled water to remove loose fibers, dried to a constant weight at 100°C, and weighed. The filters with the flagellates were rinsed with isotonic ammonium formate (0.5M) to remove sea salts and dried at 100°C for 24 h to volatilize the ammonium formate. Filters were reweighed and the concentrations of flagellates were calculated. Flagellate concentrations were determined only in the flasks with the three highest concentrations of bacteria, 5×10^7, 10^8, and 5×10^8. The biomass of bacteria was determined using the stock bacterial culture. For each species, the final biomass of flagellates (y axis) was plotted against

the initial biomass of bacteria (x axis) in the flask. The slope of this line was considered to be the yield of flagellates and its value was calculated from a simple linear regression model. The slopes for the different species were compared by ANCOVA as previously described.

The growth of P. yestita was evaluated on six bacterial isolates capable of growth on BCSF. The isolates (described in section II) included P. marina, Pseudomonas sp., Aeromonas sp., Micrococcus sp., and an unidentified gram positive rod. Exponential phase stock cultures of the bacterial isolates were inoculated into cultures containing filtered (0.2 um) York River water and 0.5 g of BCSF. Each bacterial species was cultured in triplicate. After 24 hours all the bacterial cultures had entered stationary phase and P. yestita was inoculated into the flasks. Sampling and statistical analyses were carried out as in the previous experiments.

RESULTS

All species examined were less than 10 um in length or diameter (Table III.1). The largest flagellate, P. vestita, was three times larger in length than the smallest, choanoflagellate 1, but was over 27 times larger on a volumetric basis. P. vestita was volumetrically 5 times larger than the next largest flagellate, chrysomonad 1. The two species of bodonids differed in length by 2.9 um but bodonid 1 was only 2.5 times larger than bodonid 2 on a volumetric basis.

None of the species were able to grow on BCSF in the absence of bacteria (Table III.2). No cells were observed for any species after 48 hours in the first treatment. These flasks all remained clear without any visible turbidity throughout the 48 hours, indicating a lack of bacterial growth. All flagellate species were able to achieve populations exceeding 5×10^5 cells/ml in the antibiotic control flasks. There was little difference in the concentration of flagellates between the antibiotic control and the second treatment (BCSF + bacteria), which was identical except for the addition of the antibiotic solution. For two species, the concentration of flagellates in the antibiotic control was higher than in the "BCSF + bacteria" treatment, but in all cases the flagellate concentrations were similar for the two treatments.

The growth of flagellates on bacteria in the presence of BCSF media compared favorably with their growth on bacteria resuspended in estuarine water. No pronounced differences occurred between these

Table III.1

Morphometric Characteristics of Colorless Flagellate Species.

flagellate	shape	length mean+SD (um)	width mean+SD (um)	volume (um^3)
P. vestita	sphere	7.8±0.3	---	248
chrysomonad 1	sphere	4.5±0.2	---	48
bodonid 1	ellipsoid	7.5±0.2	3.3±0.2	43
bodonid 2	ellipsoid	4.6±0.3	2.5±0.1	17
choanoflagellate 1	sphere	2.6±0.2	---	9.2

Table III.2

Flagellate Concentrations After 48 Hours Growth on BCSF, BCSF + Bacteria, Bacteria, and Antibiotic Control.

flagellate	treatment	flagellate concentration cells x 10^4/ml mean + SD, n=3
P. vestita	BCSF	< .01
	BCSF + bacteria	139 + 83.0
	bacteria	202 + 95.7
	antibiotic control	257 + 57.9
chrysomonad 1	BCSF	< .01
	BCSF + bacteria	319 + 129
	bacteria	220 + 95.4
	antibiotic control	403 + 124
bodonid 1	BCSF	< .01
	BCSF + bacteria	262 + 102
	bacteria	190 + 88.4
	antibiotic control	241 + 104
bodonid 2	BCSF	< .01
	BCSF + bacteria	398 + 74.0
	bacteria	266 + 87.2
	antibiotic control	381 + 59.6
choanoflagellate 1	BCSF	< .01
	BCSF + bacteria	61.4 + 37.8
	bacteria	104 + 65.3
	antibiotic control	51.9 + 15.6

treatments for any species. P. vestita and choanoflagellate 1 both exhibited higher cell concentrations with the resuspended bacteria while the other three species had higher cell concentrations in the presence of BCSF.

Changes in the shaking rate did not have major effects on the specific growth rates of the flagellates (Figure III.1), except for bodonid 2 which was unable to grow at 200 rpm, the highest rate of shaking. However, the growth rates of bodonid 2 at the other three speeds were not significantly different (p=0.065) when compared by ANCOVA (Table III.3). P. vestita exhibited no significant differences (p=0.083) in growth at the different shaking speeds. While ANCOVA indicated that shaking did have a significant effect on the growth rate of choanoflagellate 1, no significant differences among the levels were indicated by the Student-Newman-Keuls test (p= 0.05). No significant differences in growth rate occurred among 100, 150 and 200 rpm for both bodonid 1 or chrysomonad 1. Only for choanoflagellate 1 was there a significant difference in growth rate at each shaking speed. This was also the only species to show an increase in growth rate for each successively greater shaking rate. All other species showed a decreased growth with the greatest amount of shaking (200 rpm). All species but P. vestita showed an increase in growth at 100 rpm compared to 0 rpm. Chrysomonad 1 and bodonid 1 grew fastest at 100 rpm while bodonid 2 and P. vestita grew fastest at 150 rpm. All successive experiments used a shaking speed of 100 rpm for all species. The dissolved oxygen concentration, measured at 24 hours, in the flasks with no shaking averaged 6.1 mg/l for all species which was markedly lower than in flasks with shaking which averaged 7.8 mg/l.

Figure III.1 Effects of shaking on growth rates of five species of colorless flagellates.

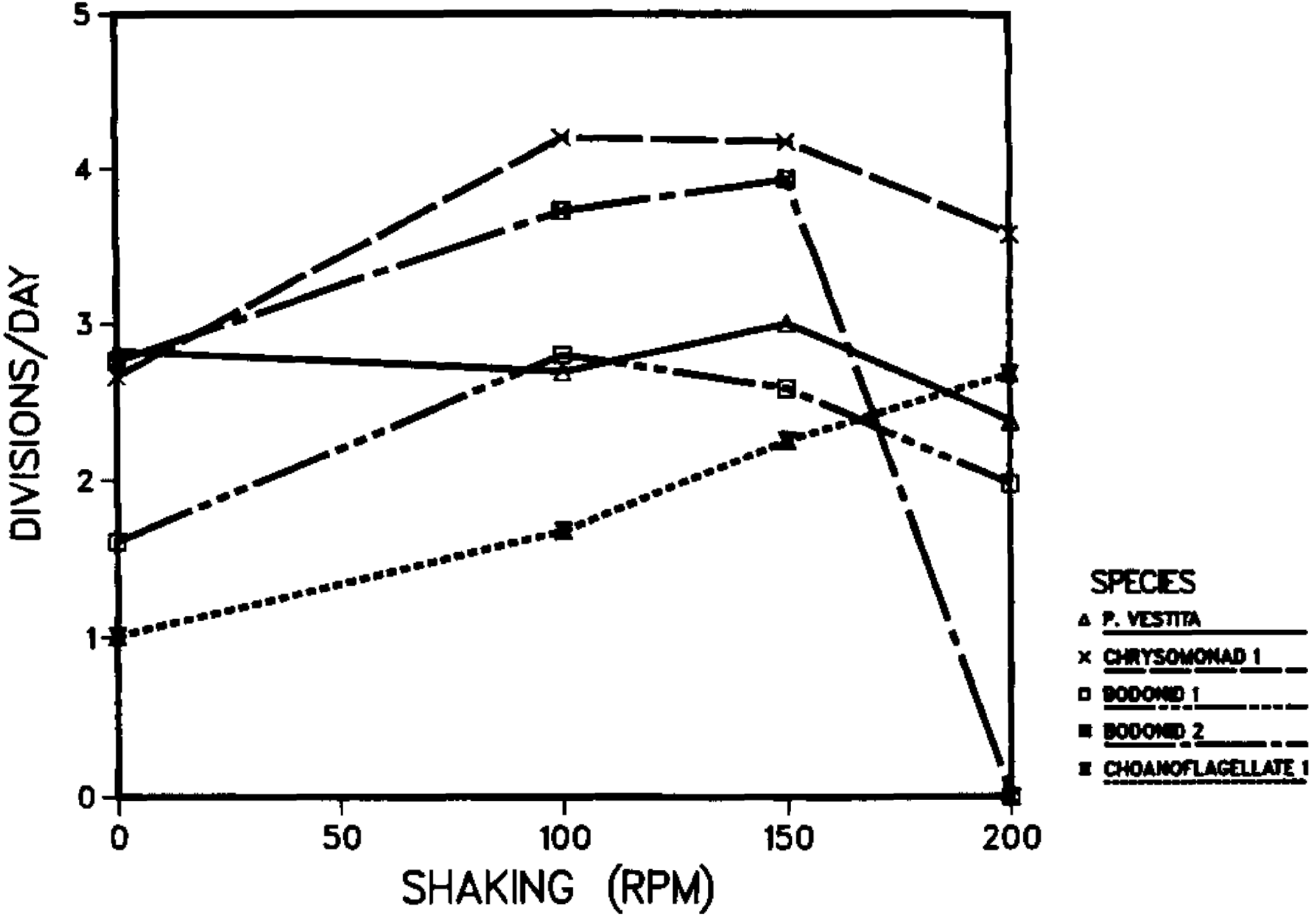

DIVISIONS/DAY
0
1
2
3
4
5
0
50
100
150
200
SHAKING (RPM)
SPECIES
P. VESTITA
CHRYSOMONAD 1
BODONID 1
BODONID 2
CHOANOFLAGELLATE 1

Table III.3

Specific Growth Rates of Colorless Flagellates at Four Shaking Speeds.

A. *Paraphysomonas vestita*

Shaking (rpm)	growth rate (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
0	0.082	2.8	.941	3.58	10
100	0.072	2.7	.978	1.18	10
150	0.087	3.0	.973	1.80	10
200	0.069	2.4	.958	1.80	10
Pooled				8.34	40
Common				9.81	—

d.f = 3,40, F = 2.355 p=0.086

B. chrysomonad 1

Shaking (rpm)	growth rate (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
0	0.077	2.7	.95	4.24	9
100	0.12	4.2	.918	11.4	10
150	0.12	4.2	.929	9.59	10
200	0.10	3.6	.956	4.27	10
Pooled				29.6	39
Common				44.5	—

d.f = 3,39, F = 6.59, p =.001

Student-Newman-Keuls test for significant differences among growth rates. Bars underline equal means (alpha = 0.05).

100 150 200 0

C. bodonid 1

Shaking (rpm)	growth rate (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
0	0.047	1.6	.890	4.65	13
100	0.081	2.8	.856	9.49	10
150	0.075	2.6	.914	8.98	13
200	0.057	2.0	.910	5.57	13
Pooled				28.7	49
Common				38.9	—

d.f = 3,49, F = 5.83, p =.0017

Table III.3 (continued)

Student-Newman-Keuls test for significant differences among growth rates. Bars underline equal means (alpha = 0.05).

100	150	200	0

D. bodonid 2

Shaking (rpm)	growth rate (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
0	0.08	2.8	.985	1.64	13
100	0.11	3.7	.861	16.2	10
150	0.11	3.9	.875	15.9	10
200	-----	---	----	---	--
Pooled				33.8	33
Common				42.2	—

d.f = 2,37, F = 2.966 p =.0638

E. choanflagellate 1

Shaking (rpm)	growth rate (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
0	0.029	1.0	.789	3.95	13
100	0.049	1.7	.933	2.89	13
150	0.065	2.3	.941	4.60	13
200	0.078	2.7	.975	2.64	13
Pooled				14.1	52
Common				36.8	—

d.f = 3,52, F = 28.0, p <.001

Student-Newman-Keuls test for significant differences among growth rates. Bars underline equal means (alpha = 0.05).

200	150	600	0

All five species of flagellates were euryhaline and able to grow at salinities ranging from 5°/oo to 35°/oo (Figure III.2). Both chrysomonad 1 and bodonid 2 showed no significant differences ($p=0.05$) in specific growth rates at any salinity (Table III.4). No significant differences in specific growth rate occurred at salinities ranging from 5°/oo to 25°/oo for P. vestita and from 15°/oo to 35°/oo for choanoflagellate 1. Chrysomonad 1 was the only species whose growth rate was less at the lowest salinity, but this difference was not significant. For P. vestita, bodonid 1, and bodonid 2 the specific growth rates decreased with increasing salinity.

Temperature had a significant effect on the specific growth rates of all species of flagellates (Table III.5). Bodonid 1 and choanoflagellate 1 exhibited maximal growth rates at 21°C while bodonid 2, chrysomonad 1 and P. vestita exhibited maximal growth rates at 26 °C (Figure III.3). The growth rates of bodonid 2 declined sharply at temperatures above and below its optimal temperature for growth, while the other species maintained high growth rates over a wide range of temperatures. The specific growth rates of all species except P. vestita declined sharply from 26°C to 31°C. P. vestita had a higher optimal temperature for growth than the other species; there was no significant difference in its growth rate between 26°C and 31°C. Growth rates for all species declined with decreasing temperatures below 21°C and were less than 1.0 divisions/day at 5°C. The growth rates of all species (except P. vestita) declined sharply at temperatures above the optimal temperature for growth and declined more gradually at temperatures below. P. vestita's growth rate may also decline sharply

Figure III.2 Effects of salinity on growth rates of five species of colorless flagellates.

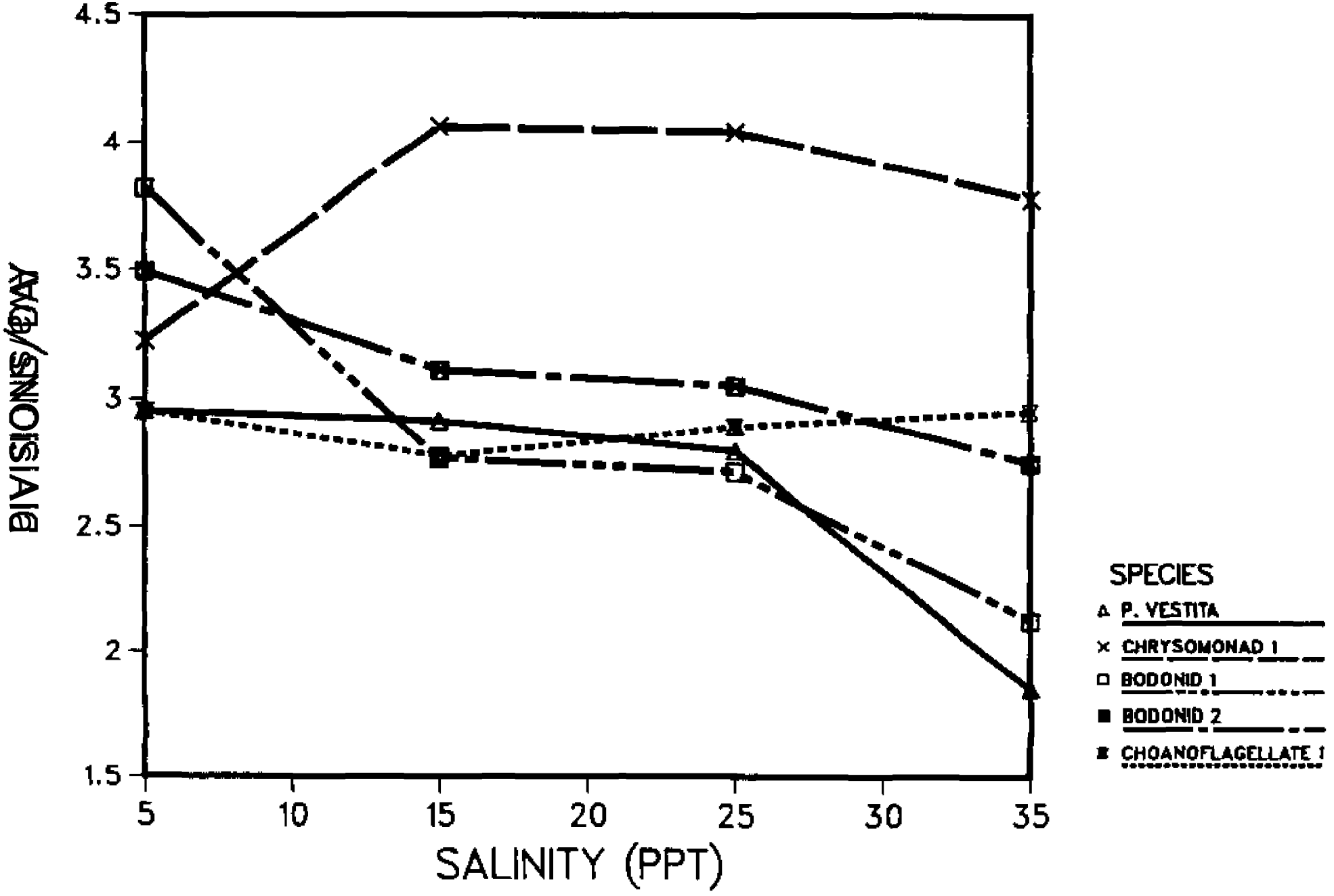

4.5
4
3.5
3
2.5
2
1.5
AVG. DIVISIONS/DAY
5
10
15
20
25
30
35
SALINITY (PPT)
SPECIES
P. VESTITA
CHRYSOMONAD 1
BODONID 1
BODONID 2
CHOANOFLAGELLATE 1

Table III.4

Specific Growth Rates of Flagellates at Four Salinities.

A. *Paraphysomonas vestita*

salinity (°/oo)	growth rate (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
5	0.085	3.0	.953	3.07	10
15	0.084	2.9	.977	1.38	10
25	0.081	2.8	.987	.710	10
35	0.053	1.8	.965	1.74	13
Pooled				6.91	43
Common				16.4	—

d.f = 3,43, F = 19.7, p <.001

Student-Neuman-Keuls test for significant differences among growth rates. Bars underline equal means (alpha = 0.05).

5 15 25 35

B. chrysomonad 1

salinity (°/oo)	growth rate (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
5	0.093	3.2	.956	14.3	9
15	0.12	4.1	.957	3.40	10
25	0.12	4.0	.972	5.32	10
35	0.11	3.8	.876	3.32	8
Pooled				26.3	37
Common				29.5	—

d.f = 3,43, F = 2.68, p =.059

C. bodonid 1

salinity (°/oo)	growth rate (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
5	0.11	3.8	.954	4.80	10
15	0.080	2.8	.936	6.06	10
25	0.078	2.7	.968	3.48	10
35	0.061	2.1	.973	1.68	13
Pooled				16.0	43
Common				29.1	—

d.f = 3,43, F = 11.7, p <.001

Table III.4 (continued)

Student-Newman-Keuls test for significant differences among growth rates. Bars underline equal means (alpha = 0.05).

5	15	25	35

D. bodonid 2

salinity (°/OO)	growth rate (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
5	0.10	3.5	.924	6.75	7
15	0.090	3.1	.941	8.71	13
25	0.088	3.1	.931	10.0	13
35	0.079	2.7	.984	1.71	13
Pooled				6.90	46
Common				29.9	—

d.f = 3,52, F = 2.02, p = 0.122

E. choanoflagellate 1

salinity (°/OO)	growth rate (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
5	0.024	1.2	.876	1.13	10
15	0.080	2.8	.955	5.20	13
25	0.084	2.9	.908	12.2	13
35	0.085	3.0	.954	5.97	13
Pooled				24.5	49
Common				64.5	—

d.f = 3,49, F = 26.6, p <.001

Student-Newman-Keuls test for significant differences among growth rates. Bars underline equal means (alpha = 0.05).

35	25	15	5

Table III.5

Specific Growth Rates of Flagellates at Five Temperatures.

A. Paraphysomonas vestita

temperature (°C)	growth rate (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
5	0.013	0.46	.058	24.8	10
13	0.038	1.3	.369	43.8	13
21	0.074	2.6	.966	3.36	13
26	0.098	3.9	.963	3.22	10
31	0.094	3.3	.753	25.3	10
Pooled				101.	56
common				277.	—

d.f = 4,56, F = 7.34, p <.001

Student-Newman-Keuls test for significant differences among growth rates. Bars underline equal means (alpha = 0.05).

26 31 21 13 5

B. chrysomonad 1

temperature (°C)	growth rate (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
5	0.020	0.68	.115	50.8	13
13	0.087	3.0	.908	13.2	13
21	0.13	4.6	.948	8.29	10
26	0.13	4.6	.928	12.1	10
31	0.021	.72	.154	19.2	7
Pooled				104.	53
Common				241.	—

d.f = 4,53, F = 17.6, p <.001

Student-Newman-Keuls test for significant differences among growth rates. Bars underline equal means (alpha = 0.05).

26 21 13 31 5

Table III.5 (continued)

C. bodonid 1

temperature (°C)	growth rate (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
5	0.020	0.71	.670	3.12	10
13	0.058	2.0	.991	.467	10
21	0.11	3.8	.992	.694	7
26	0.10	3.5	.995	.366	7
31	0.052	1.8	.947	2.27	10
Pooled				6.92	44
Common				64.2	—

d.f = 4,44, F = 91.1, p <.001

Student-Newman-Keuls test for significant differences among growth rates. Bars underline equal means (alpha = 0.05).

21	26	13	31	5

D. bodonid 2

temperature (°C)	growth rate (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
5	0.022	0.76	.843	1.54	13
13	0.052	1.8	.944	2.78	13
21	0.12	4.1	.982	2.30	10
26	0.18	6.1	.993	.725	7
31	0.067	2.3	.954	3.75	13
Pooled				10.9	56
Common				112.	—

d.f = 4,44, F = 91.1, p <.001

Student-Newman-Keuls test for significant differences among growth rates. Bars underline equal means (alpha = 0.05).

26	21	31	13	5

Table III.5 (continued)

E. choanoflagellate 1

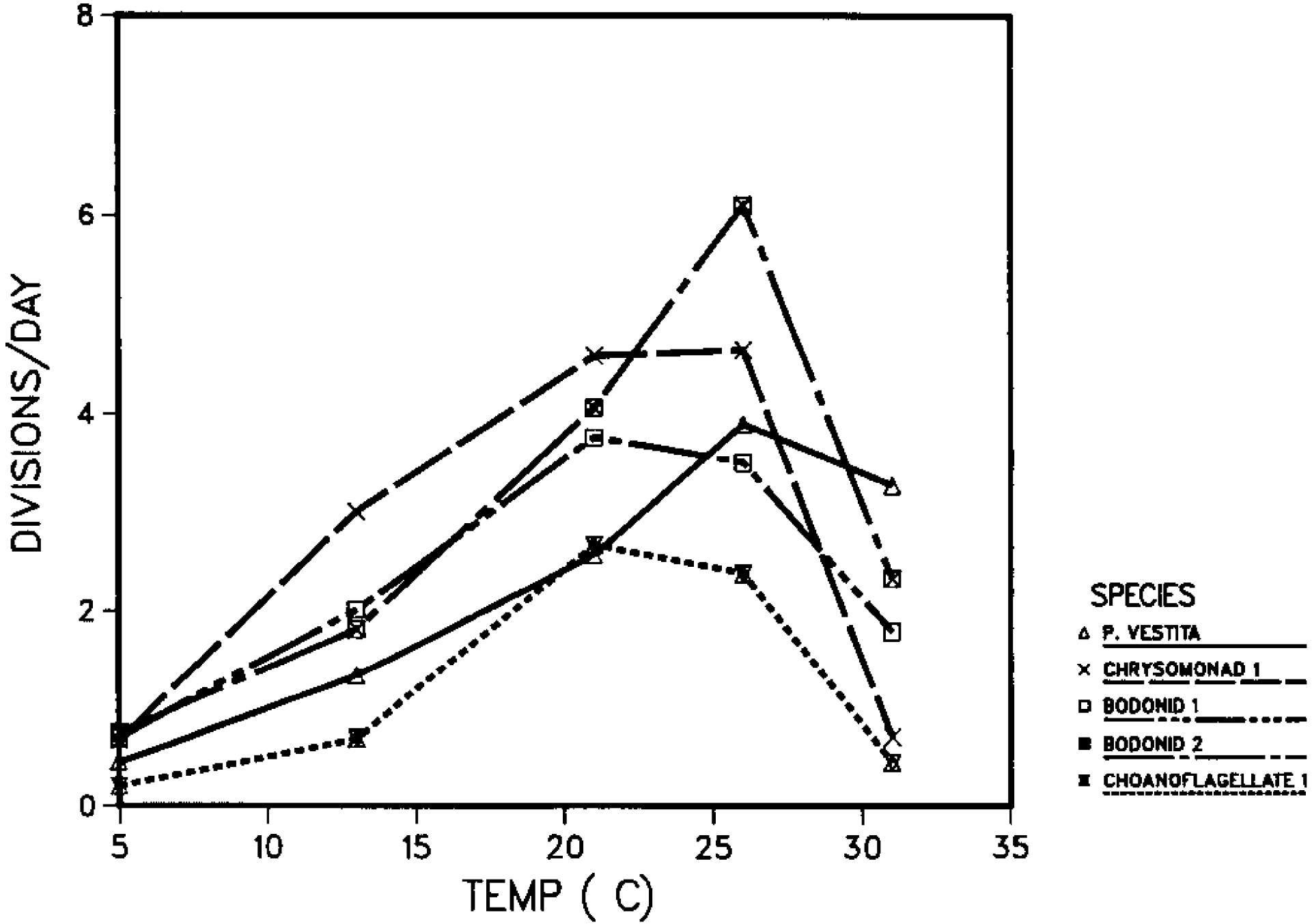
temperature (°C)	growth rate (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
5	0.0060	0.21	.740	.222	13
13	0.020	0.70	.981	.141	13
21	0.077	2.7	.973	2.84	13
26	0.069	2.4	.952	4.10	13
31	0.013	0.46	.880	.408	13
Pooled				7.71	65
Common				84.3	—

d.f = 4,65, F = 161., p <.001

Student-Newman-Keuls test for significant differences among growth rates. Bars underline equal means (alpha = 0.05).

21 26 13 31 5

Figure III.3 Effects of temperature on growth rates of five species of colorless flagellates.


DIVISIONS/DAY
0
2
4
6
8
5
10
15
20
25
30
35
TEMP (C)
SPECIES
P. VESTITA
CHRYSOMONAD 1
BODONID 1
BODONID 2
CHOANOFLAGELLATE 1

above the optimal temperature but sufficiently high temperatures were not tested.

Flagellate growth rates at four concentrations of the bacterium Pseudomonas marina are given in Table III.6a. The specific growth rates of P. vestita, chrysomonad 1 and bodonid 1 increased for each successively higher concentration of bacteria, while the specific growth rates of bodonid 2 increased only up to 100 x 10^6 bacteria/ml and then declined at 500 x 10^6 cells/ml, the highest bacterial concentration. Although the specific growth rates increased with increasing bacterial concentration, they were not always significantly different. No significant differences occurred among the growth rates at 50, 100, and 500 X 10^6cells/ml for P. vestita and no significant difference occurred between 50 and 100 X 10^6cells/ml for chrysomonad 1. Only bodonid 1 showed a significant difference at each successively higher concentration of bacteria.

The response of a microorganism's growth rate to changes in a limiting nutrient can be modeled by a hyperbolic function which is a characteristic of Michaelis-Menten kinetics (Pirt, 1975). If the bacterial concentration is limiting, such a response can be described by the equation, $u=u_m s/(s+K_s)$ where u is the specific growth rate (h^{-1}) at a bacterial concentration s, u_m is the maximum specific growth rate, and K_s is the bacterial concentration at which the specific growth rate is one half of the maximum. When the specific growth rates of the flagellates are plotted against a linear scale of bacterial concentration, saturating curves suggestive of Michaelis-Menten kinetics result (Figure III.4). Sufficient data to statistically test the fit of such a model was not obtained but approximate values for the parameters

Table III.6a

Specific Growth Rates of Flagellates at Four Concentrations of Bacteria.

A. Paraphysomonas vestita

bacteria ($X 10^6$/ml)	growth (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
10	0.078	2.7	.967	1.76	10
50	0.093	3.2	.969	2.39	10
100	0.10	3.5	.974	2.36	10
500	0.11	3.8	.985	1.53	10
Pooled				8.03	40
Common				12.7	—

d.f = 3,40, F = 7.81, p <.001

Student-Newman-Keuls test for significant differences among growth rates. Bars underline equal means (alpha = 0.05).

500 100 50 10

B. chrysomonad 1

bacteria ($X 10^6$/ml)	growth (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
10	0.045	1.5	.782	9.59	13
50	0.11	4.0	.985	1.65	10
100	0.12	4.2	.977	3.04	10
500	0.14	5.0	.986	2.66	10
Pooled				16.9	43
Common				91.2	—

d.f = 3,43, F = 62.8, p <.001

Student-Newman-Keuls test for significant differences among growth rates. Bars underline equal means (alpha = 0.05).

500 100 50 10

Table III.6a (continued)

C. bodonid 1

bacteria ($X 10^6$/ml)	growth (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
10	0.065	2.2	.924	2.98	10
50	0.085	3.0	.981	1.21	10
100	0.10	3.5	.986	1.26	10
500	0.12	4.1	.991	1.15	10
Pooled				6.60	40
Common				20.5	—

d.f = 3,40, F = 28.1, p <.001

Student-Newman-Keuls test for significant differences among growth rates. Bars underline equal means (alpha = 0.05).

500	100	50	10

D. bodonid 2

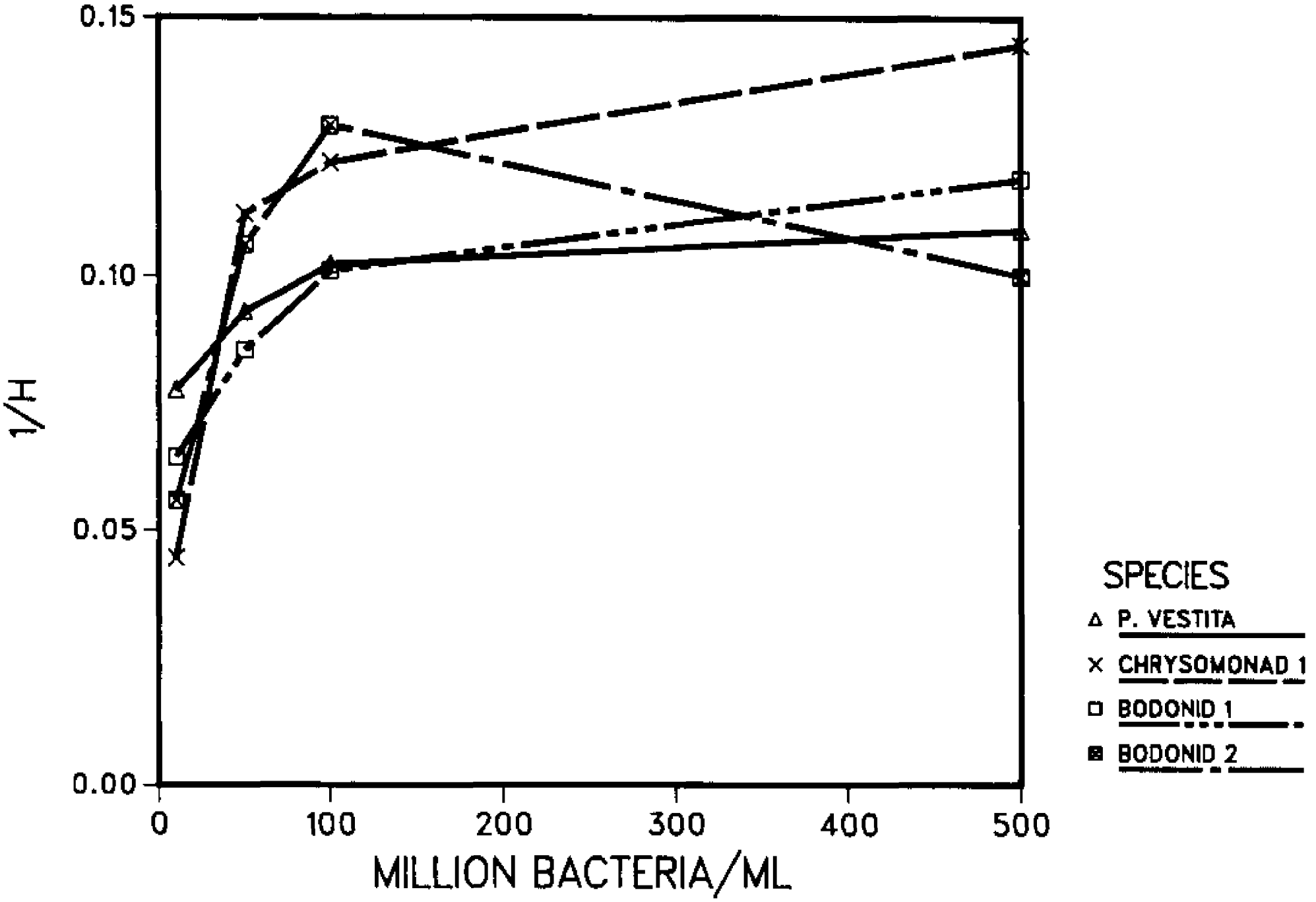
bacteria ($x10^6$/ml)	growth (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
10	0.056	1.9	.886	3.48	10
50	0.11	3.7	.974	2.56	10
100	0.13	4.5	.987	1.90	10
500	0.10	3.4	.965	3.13	10
Pooled				11.1	40
Common				35.5	—

d.f = 3,40, F = 29.4, p <.001

Student-Newman-Keuls test for significant differences among growth rates. Bars underline equal means (alpha = 0.05).

100	50	500	10

Figure III.4 Effects of bacterial concentration on growth rates of four species of colorless flagellates.


0.15
0.10
0.05
0.00
1/H
0
100
200
300
400
500
MILLION BACTERIA/ML
SPECIES
P. VESTITA
CHRYSOMONAD 1
BODONID 1
BODONID 2

were estimated by linear regressions of double reciprocal transformations of the data, a Lineweaver-Burke transformation (Table III.6b). Data obtained from batch cultures are biased because nutrient concentrations do not remain constant, but they can still be used to calculate approximations of the true kinetic parameters (Bailey and Ollis, 1981). The specific growth rate at 50 X 10^7cells/ml was not included in calculating the kinetic parameters for bodonid 2. The largest u_m was obtained with chrysomonad 1, followed by bodonid 2, bodonid 1,and P. vestita. K_s values showed the same rank order.

For each species of flagellate, the number of cells produced was linearly related to the initial concentration of bacteria (Figure III.5). Yield for each flagellate (Table III.7) was calculated from the slope of the line relating the final concentration of each flagellate (dry weight) to the initial concentration of bacteria (dry weight). The lines all had correlation coefficients exceeding 0.90, but each was calculated from only three data points. Yields ranged from 0.30 for bodonid 1 to 0.42 for chrysomonad 1. The Student-Newman-Keuls test indicated that only the largest and smallest yields were just significantly different at the 5% significance level.

The specific growth rates of P. vestita, when cultured on different isolates of bacteria, ranged from 0.10 h^{-1} on Aeromonas sp. to 0.073 h^{-1} on Micrococcus sp. (Table III.8). Although there were significant differences in the specific growth rates on the different bacterial diets, the Student-Newman-Keuls test indicated that no significant differences existed among the top four (Aeromonas sp., P. marina, Pseudomonas sp., and the gram positive rod) diets giving the highest growth rates. The three poorest diets (the gram positive rod,

Table III.6b

Growth Kinetic Parameters of Flagellates Cultured on *Pseudomonas marina*.

flagellate	μ (h^{-1})	K_s (X 10^7/ml)	r^2
P. vestita	0.105	3.66	.969
chrysomonad 1	0.158	25.3	.998
bodonid 1	0.108	6.98	.957
bodonid 2	0.144	15.9	.998

Figure III.5 Effects of bacterial concentration on yields of four species of colorless flagellates.

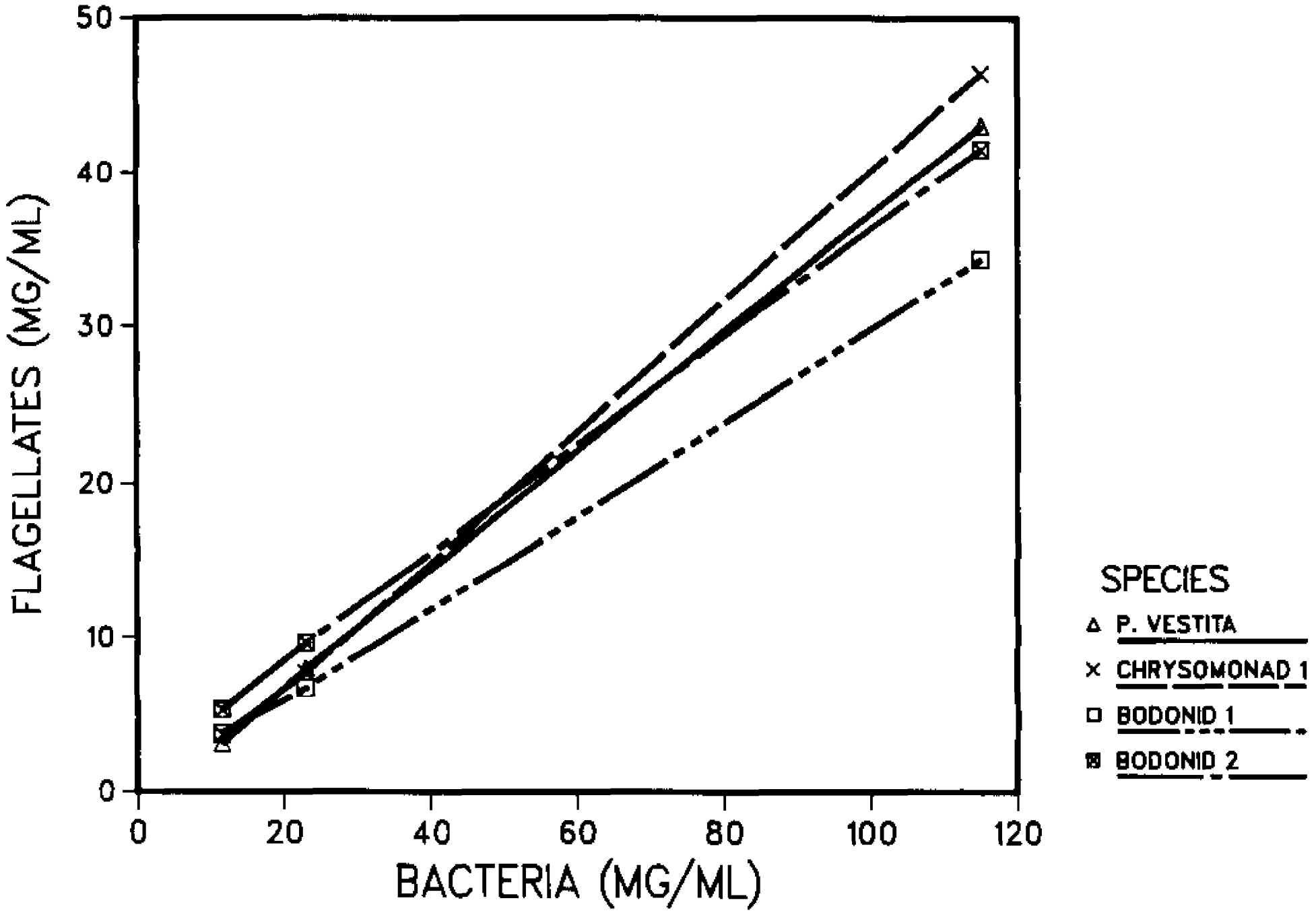

50
40
30
20
10
0
FLAGELLATES (MG/ML)
0
20
40
60
80
100
120
BACTERIA (MG/ML)
SPECIES
P. VESTITA
CHRYSOMONAD 1
BODONID 1
BODONID 2

Table III.7

Yields of Flagellates Cultured on *Pseudomonas marina*.

flagellate	yield	correlation coefficient	residual S.S.	residual d.f.
P. vestita	0.38	0.98	57.9	7
chrysomonad 1	0.42	0.98	53.3	7
bodonid 1	0.35	0.91	160.	7
bodonid 2	0.30	0.97	61.4	7
pooled			332.	28
common			483.	--

d.f. = 3,28, F = 4.22, p = 0.014

Student-Newman-Keuls test for significant differences among yields. Bars underline equal means (alpha = 0.05).

chrysomonad 1 *P. vestita* bodonid 2 bodonid 1

Table III.8

Specific Growth Rates of *Paraphyaomonas vestita* on Six Isolates of Bacteria.

bacterial isolate	growth rate (h^{-1})	divisions day^{-1}	correlation coefficient	residual SS	residual d.f.
P. marina	0.093	3.2	.997	.426	13
Pseudomonas sp.	0.090	3.1	.993	1.02	13
Aeromonas sp.	0.10	3.5	.997	.226	10
Flavobacter sp.	0.077	2.7	.995	.508	13
Micrococcus sp.	0.073	2.5	.966	3.25	13
Gram+ rod	0.087	3.0	.946	7.46	13
Pooled				12.9	75
Common				20.17	—

d.f = 5,75, F = 8.38, p <.001

Student-Newman-Keuls test for siginificant differences among growth rates. Bars underline equal means (alpha = 0.05).

Aeromonas *P. marina* *Pseudomonas sp.* gram+ rod *Flavobacter* *Micrococcus*

Flavobacter sp., *Micrococcus* sp.) were likewise not significantly different.

DISCUSSION

The inability of the flagellates to grow on BCSF in the absence of bacteria was expected. Similar strains of colorless flagellates have been demonstrated to have a phagotrophic mode of nutrition. Brooker (1971) described the functional morphology of bacterial ingestion in two species of bodonids and found vacuoles containing bacteria in the cells. Haas and Webb (1979) examined five species of flagellates and found them unable to take up 11 different organic substrates in solution including amino acids, glycerol, glucose and acetate. Electron micrographs demonstrated that the flagellates, which included P. vestita, two other chrysomonads, a bodonid and an amphimonad, were ingesting bacteria. Fenchel (1982a) studied two chrysomonads (including P. vestita), a bicoecid, a bodonid, a choanoflagellate, and a helioflagellate and found them all to be phagotrophic.

Some free-living protozoa, however, have been cultured axenically on soluble nutrients. The choanoflagellates Diaphanoeca grandis and Acanthoecopsis sp. have been cultured on a mixture of acetate, liver concentrate, and vitamins (Gold, et al., 1970). Two strains of the colorless dinoflagellate Crypthecodinium cohnii have been grown in simple defined media with a variety of carbon sources including glucose, glycerol, ethanol, and various organic acids (Provasoli and Gold, 1962; Gold and Barren, 1966). Oxyrrhis marina, a brackish water dinoflagellate can be cultured on a defined medium with either acetate or ethanol as a carbon source (Droop, 1970). Marine ciliate genera

cultured on defined media include Uronema (Hanna and Lilly, 1972; Lee et al., 1971), Miamiensis, and Parauronema (Soldo and Merlin, 1972). Peranema, a colorless euglenoid, has been raised in an axenic culture on a milk-lecithin medium (Allen et al., 1966).

In all these examples, however, the protozoa were also capable of growth on a bacterial diet. Protozoa raised on dissolved nutrients typically grow slower than those fed bacteria (Sleigh, 1973). When cultured on dissolved nutrients, much higher carbon concentrations may be required than when cultured phagotrophically. Fenchel (1982a) calculated that Ochromonas growing on bacteria was 2 X 10^4 times more efficient than when cultured on soluble nutrients.

The lack of flagellate growth on BCSF in the absence of bacteria may reflect a true inability to grow osmotrophically or it may result from an inability to utilize the organic compounds in the BCSF. BCSF did not appear lo supplement the growth of flagellates even in the presence of bacteria. The ability to use mono- and disaccharides is common in free-living protozoa but the ability to use any particular sugar varies from species to species (Hall, 1965). Maltose, a major component of BCSF, can be used by a large number of ciliates and some flagellates (Hall, 1965). Sucrose, another dissacharide in BSCF, can be used by many protozoa as well.

Another explanation for the inability of the flagellates to grow on BCSF is the lack of necessary vitamins or growth factors. Bacteria, cultured on BCSF, might produce the micronutrients necessary for the flagellates. Kinne (1977) summarized the vitamin requirements for marine protozoa. Free-living flagellates, in general, require thiamine, B_{12} , riboflavin, and biotin. Other specific micronutrients may be

required by particular species or strains of protozoa. Examples include alpha lipoic acid by Tetrahymena pyriformis, ascorbic acid by trypanosomatid flagellates, choline by Trichomonas gallinae, quinones by Oxyrrhis marina and sterols by Paramecium aurelia. All of these dietary requirements may be provided by bacteria when the protozoa are feeding in bacterized cultures. Spent yeast, one of the components from which BCSF is manufactured is a rich source of vitamins, but no information is currently available on the vitamin levels in BCSF itself. The high temperature at which BCS is produced may result in the degradation of vitamins originally present.

While flagellates may not be able to grow directly on BCSF, it was not toxic to them since the growth of flagellates cultured on resuspended bacteria in York River water did not differ greatly from the growth of those cultured on bacteria in the presence of BCSF. The small quantities of ethanol in the BCSF are probably not inhibitory to flagellates. Ukeles and Rose (1976) found that 10 short chain alcohols (C_1 to C_4) had no effect on four species of chrysomonads and one cryptomonad; only long chain compounds were toxic or inhibitory.

Shaking rate influenced both the amount of dissolved oxygen in the culture and the degree of physical agitation. It is difficult to determine the relative significance of these factors on the growth rates of the flagellates. Four flagellate species exhibited an increase in growth rate between 0 rpm and 100 rpm, which may be related to the slight increase in dissolved oxygen at 100 rpm. Fenchel (1982b) grew six similar species of flagellates at comparable cell densities and found no differences between stationary and shaken cultures. Respiration rates, in Fenchel's study, were on the order of 5×10^{-9} mg

oxygen/cell/h. A culture of 10^6 flagellates/ml would consume 5×10^{-3} mg oxygen/ml/h. In addition, the bacteria would also contribute to the oxygen demand of the culture. A stationary culture, dependent only on diffusion from the air could realize a significant drop in oxygen levels, especially if the surface of the culture exposed to the air was small relative to its depth.

Little is known about how reduced oxygen concentrations affect the growth of estuarine flagellates. There are species of flagellates which can survive and reproduce under anaerobic conditions and other aerobic species which can tolerate reduced oxygen levels for short periods (Curds, 1975). Species of chrysomonads, euglenoids, bicoecids, bodonids, and diplomonads have all been reported to survive in anaerobic waste treatment facilities. The extent to which the species used in this study can tolerate extremely low dissolved oxygen conditions is unknown.

The reduction in growth rates at 0 rpm could also be attributed to the physical agitation of the cultures which might be preventing the flagellates or bacteria from settling out. Mixing processes which enhance contact between the flagellates and their bacterial prey could be stimulatory to the growth rates. Increased agitation due to shaking, rather then any oxygenation effects, may have decreased the growth rates at 200 rpm. Bodonid 2 was unable to grow at the highest speed and three other species had reduced growth rates. Although not quantified, there appeared to be a larger number of flagellate cells missing flagella at the highest shaking speed. The shear forces developed at this speed might be stripping flagella from the cells (Blum, 1971). Bodonid 2 might be more susceptible to this effect because it is a small cell (4 u

in length) with an extremely long trailing flagella (12-15 u). Another possibility is that physical aggitation is interfering in feeding processes of the flagellates. At none of the speeds did biomass accumulate on the walls of the flask above the liquid level as a result of splashing. This was a problem in preliminary experiments in which flagellates were cultured on a reciprocal shaker.

All five species of flagellates are euryhaline and able to grow at salinities ranging from 5 °/oo to 35 °/oo and only one, choanoflagellate 1 showed a significantly reduced growth rate at the lowest salinity tested. This was not surprising since they were all isolated from the lower York River at Gloucester Point, VA. The salinity in the lower York River ranges from 10°/oo to 25°/oo (Brooks, 1983) depending on the time of year and amount of freshwater runoff. In addition to seasonally differing salinities, flagellates in the river may be exposed to large salinity changes as they are cycled within the estuary. Wide salinity tolerances are characteristic of estuarine phytoplankton (Rice and Ferguson, 1975).

The flagellates in this experiment were inoculated from a stock culture at 17°/oo into the cultures at different salinities without any preconditioning. Acclimation to a given salinity has been demonstrated for various phytoflagellates to shift the high and low salinity tolerances as well as shift the optimum range (Sorge and McLaughlin, 1971; Mahoney and McLaughlin, 1979). Since salinity limits for the species tested were not determined, it is difficult to predict if preconditioning would have any effects on growth.

Flagellates respond to salinity in a manner similar to estuarine dinoflagellates, i.e. both exhibit growth rates close to maximum over a

wide range of salinities (Braarud, 1961; White, 1978). Dinoflagellates typically have maximum growth rates at 15 to 20°/oo and have significantly reduced growth at 5°/oo. The flagellates, however, had high growth rates at 5°/oo, a salinity at which many estuarine dinoflagellates cannot survive. The flagellates in this study may be similar to some estuarine phytoflagellates which are capable of high growth rates at even the lowest salinities. The chrysomonad Olisthodiscus luteus can grow in salinities ranging from 2°/oo to 50°/oo (Tomas, 1978). Dunaliella tertiolecta, a chlorophyte has been cultured at salinities ranging from 3.7°/oo to 120°/oo (Mclachlan, 1960) and another chlorophyte, Chlamydomonas pulsatilla, shows significant growth from 0.05°/oo to 100°/oo, with high growth rates occurring up to 60°/oo (Hellebust and Le Gresley, 1985).

Some flagellates in this study may be identical to strains of protozoa which are considered to be freshwater species. P. vestita, which has been isolated from freshwater (Takahashi, 1976), can withstand salinities ranging from distilled water to 91°/oo (Lee, 1978). In a study of protozoa from Wisconsin, Finley (1930) found that some species of flagellates, such as Bodo uncinatus, are capable of surviving and reproducing when transferred directly to seawater. Other species, such as Monas sp., could grow well in seawater but required a gradual increase in salinity.

Of the environmental variables tested, growth of the flagellates was influenced most strongly by temperature. Although differences in apparent optimal temperature occurred among the five species, they were all eurythermal. For each species, the growth rate exceeded half the maximum growth rate for a temperature range exceeding 10°C. Organisms

found in temperate estuaries are exposed to large temperature variations and usually have wide temperature tolerances. Temperatures in the York River vary seasonally from -1°C to over 30°C. Various phytoplankton species from estuarine and coastal waters have wide temperature tolerances and broad optimal temperatures for growth (Ukeles, 1961). The optimal temperatures for growth in this study were in the range of 21°C to 26°C, except for P. vestita. This is the temperature range in which many species of phytoplankton exhibit their maximal growth rates (Eppley, 1972).

The overall response to temperature was very similar for the five species; growth rates declined sharply at temperatures above the optimal temperature for growth and declined even more gradually at temperatures below the optimum. This response is characteristic of a large number of marine and freshwater algae (Eppley, 1972; Goldman and Carpenter, 1974). Payer et al. (1980) examined the growth of 34 strains of phytoplankton in laboratory cultures and found this response in the majority of species. Cloern (1977) observed this response to temperature in Cryptomonas ovata and found that it could be described by an exponential function which incorporates growth rate at a basal temperature and upper lethal temperature among its parameters. Similarly shaped curves are characteristic of heterotrophic growth. The growth rate of Euglena gracilis, cultured on sodium acetate as a sole carbon and energy source, increased gradually at temperatures up to 29°C and then declined sharply at higher temperatures.

The asymmetry of this growth response should be considered when deciding upon an operating temperature for large scale culture of flagellates for a mariculture operation. For a flagellate, such as

bodonid 2 being raised at its optimal temperature, an accidental rise in temperature of 5°C could cause the culture to crash. If it were cultured at a temperature 4 or 5°C lower, it would be less affected by temperature variations with only a small decrease in biomass production.

Michaelis-Menten kinetics seem to describe the response of flagellate growth to food supply in the present study. In a study of six species of colorless flagellates, Fenchel (1982) also found that Michaelis- Menten kinetics could accurately describe the relationship between growth rate and bacterial concentration. Most of the species had growth rates close to maximum at bacterial concentrations between 5 x 10^7 and 10^8 cells/ml, although one species, Actinomonas mirabilis, had growth rates close to maximum at 10^7cells/ml. These concentrations are all similar to those at which maximum growth rates were recorded in this study. The K_s values, as well, were similar to those in this study.

Both kinetic parameters, K_s and u_m, may influence the choice of a flagellate species to be used in a mariculture operation. When the objective is to maximize the production of flagellate biomass, a species with a high u_m will obviously produce more biomass in a given time than a species with a low u_m at saturating bacterial concentrations. If two species have the same u_m, the species with the low K_s will outproduce the one with the high K_s if bacterial concentrations are not saturating. A species with a low u_m may even outproduce a species with a high u_m at non-saturating bacterial concentrations if the K_s is sufficiently low. A knowledge of the kinetic parameters of the flagellate species will be important when optimizing production of flagellate biomass.

P. vestita had a maximum specific growth rate (u_m) of 0.11 h^{-1} which is close to that of the other species, but its saturation constant (K_s) was 0.37 X 10^6cells/ml which was lower than the other species. These estimates may be biased because P. vestita had a growth rate close to maximum at the lowest bacterial concentration tested, 10^7cells/ml. This reduces the accuracy of the Linneweaver-Burke transformations in estimating the kinetic parameters (Grady and Lim, 1980). With the same strain of P. vestita, Fenchel found that it approached a maximum growth rate at 5 X 10^7cells/ml and had a K_s of approximately 10^7cells/ml . This discrepancy may not entirely be due to bias in the estimates of P. vestita's kinetic parameters. Other researchers have found that kinetic parameters are affected by environmental conditions such as temperature or by differences in biomass and nutritional value of the bacteria. Rivier et al. (1985) examined growth of Pseudobodo sp. in four experiments using natural assemblages of bacteria, which varied from experiment to experiment, and found values of K_s ranging from 0.648 X 10^7 to 2.04 X 10^7cells/ml. Similarly, Sherr et al. (1983) found very differently shaped curves when Monas sp. was cultured on four species of bacteria over the same range of bacterial concentrations on a dry weight basis.

Yields of flagellates in this study, which ranged from 30% to 42%, were comparable to those documented by other researchers. Such comparisons, however, are only approximations since yields are frequently calculated in different ways and with different units (cells, percentage carbon, biovolume), without the necessary conversion factors to put them in common units. Fenchel (1982b) reported yields based on carbon content of Ochromonas sp. and Pleuromonas jaculans of 34% and 43%

respectively when cultured on Pseudomonas. P. vestita, in the same study, had a yield of 9.1 X 10^{-4} cells/bacteria, which is equivalent to a yield of 29%, based on the reported volumes of the flagellates and bacteria. Paraphysomonmas imperforata was reported to have a yield of 44%, based on volume, when cultured on either a bacterial diet or on the diatom Phaeodactylum tricornutum (Caron et al., 1985). Yields of Monas sp. ranged from 23.7% to 45% depending on the species of bacteria used for food (Sherr et al., 1983).

The six bacterial isolates examined as foods for flagellates all supported growth of P. vestita but they differed in nutritional value. Other studies have also demonstrated that bacteria may differ in nutritional value to a given protozoan. Hardin (1944) found that of 30 bacterial isolates tested, 25 permitted growth of the flagellate Oikomonas termo, but were of varying food value. Sherr et al. (1983) found that the freshwater flagellate Monas sp. had significantly different growth rates when fed four species of bacteria, yet the growth efficiencies on the different bacteria were all equal. Other studies have demonstrated qualitative differences in bacterial prey for ciliates (Barna and Weiss, 1973; Taylor, 1978), amoebae (Groscop and Brent, 1966; Heal and Felton, 1970), and flagellates (Singh, 1942; Gorjacheva et al., 1978). The underlying reasons for these nutritional differences are poorly understood. They may be related to bacterial size, motility, proximate composition, cell surface composition, digestability, vitamin concentration, and toxin production. These factors, which are not mutually exclusive, have never been systematically examined for any single species of protozoan.

Production of toxins by certain strains of bacteria is one factor which has been well documented (Groscop and Brent; Heal and Felton, 1970). These toxins are frequently a pigment produced by the bacteria. Two bacterial strains used in this study, Flavobacter sp. and Micrococcus sp., which produce pigmented colonies, resulted in the poorest flagellate growth. Dive et al. (1974) examined pigments produced by these same genera but found them nontoxic to protozoa. Grosscop and Brent (1964) examined growth of five strains of amoebae fed three strains of pigment producing Flavobacter and found only one example of the bacteria being toxic. There were significant differences among the bacterial species in supporting growth of amoebae. The one species of Micrococcus examined, which also produced a pigment, supported growth of all strains of amoebae.

There is some evidence to suggest that gram negative bacteria in general may be nutritionally superior to gram positive bacteria (Barna and Weiss, 1973; Dive, 1973), but this finding, even if widespread, may be related to several of the previously mentioned factors. Proximate composition, cell surfaces and toxin production, in particular differ fundamentally between gram positive and gram negative bacteria. In this study, only two gram positive bacteria were examined and they were among the three poorest bacterial diets. A much larger sampling of bacterial species and flagellates would be required to draw any conclusions.

If the highest specific growth rates recorded for each flagellate species are compared within the shaking, salinity, temperature, and bacterial concentration experiments, the overall fastest growing flagellate was chrysomonad 1, followed by bodonid 2, bodonid 1, P. vestita, and choanoflagellate 1. In the shaking, salinity, and

bacterial concentration experiments, chrysomonad 1 had the highest growth rate of any species. In the temperature experiment bodonid 2 had a much faster growth rate (6.10 divisions/day) than did chrysomonad 1 (4.64 divisions/day). Bodonid 2, however, had a higher optimal temperature for growth with a narrower temperature range than did chrysophyte 1 . The shaking, salinity, and bacterial concentration experiments were all performed at 20°C, a temperature at which the growth rate of chrysomonad 1 was greater than that for bodonid 2. If these experiments had been performed above 26°C then bodonid 2 might have achieved the highest growth rates of any species. P. vestita had the highest optimal temperature for growth and it might have had a considerably higher growth rate if the temperatures in the experiments was above 26°C. The growth rates of bodonid 1 and choanoflagellate 1 were close to their maximum in these experiments as they were tested at temperatures close to their optimal growth temperatures.

Choanoflagellate 1, the slowest growing flagellate, might have had higher growth rates in the salinity, temperature, and bacterial concentration experiments if it had been tested at a higher rate of shaking. These experiments were run at 100 rpm, while it had a greater growth rate at 200 rpm. Changing the salinity of the experiments from 17°/oo, which is close to its optimum, would not have increased its growth rates.

Bodonid 1 was tested in the shaking, temperature, and bacterial concentration experiments at a salinity of 17°/oo which is considerably higher than 5°/oo, the salinity at which its highest growth rate was recorded. It might have achieved higher growth rates in these experiments if they were performed at a lower salinity. A shaking speed

of 100 rpm and a temperature of 20°C, however, appear to be close to optimal for this species.

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SECTION IV

Growth of Bivalves

INTRODUCTION

Brewers condensed solubles (BCS), a by-product of the brewing industry, is a plentiful and cheap source of underutilized nutrients. This study investigated the use of BCS for shellfish mariculture. BCS has been demonstrated to be adequate for culture of bacteria (section II), which can be used to raise colorless flagellates (section III). This section examines the growth of juvenile oysters (Crassostrea virginica) and clams (Mercenaria mercenaria) on bacteria, colorless flagellates, and an enrichment culture, all cultured on BCS. Feeding of BCS directly to bivalves was not examined in this study. BCS is primarily soluble and bivalves are not able to obtain sufficient nutrients for growth on a diet composed entirely of dissolved organic matter (Jorgensen, 1966).

Juvenile bivalves were chosen for these experiments because their production is often a major constraint to the financial success of a shellfish hatchery. Larval bivalve demands for food and water are miniscule when compared to those of an equal number of juveniles. Food requirements for juveniles increase geometrically with their size. Large juveniles have greatly increased survival when placed in semi-protected or unprotected plots in the natural environment (Castagna, 1984; Eldridge et al., 1979; Krantz, 1982), which makes them more

desirable to shellfish growers. The additional quantities of food and space required to produce large juveniles is reflected in their correspondingly greater cost.

In many hatcheries animals are fed monocultures of micro-algae, the production of which can represent a significant portion of the total operating cost (Bolton, 1982). Cost estimates for producing a kilogram (dry wt) of micro-algae have ranged from $44 (Bolton, 1982) to $200 (Walne, 1976). Those hatcheries using only raw water to feed animals will have greater pumping costs to raise large animals. The costs of pumping raw water to feed shellfish is comparable to that of raising micro-algae (Krantz, 1982). Any source of food that is more cheaply produced and can be substituted for a microalgal diet, will increase a hatchery's profit margin.

MATERIALS AND METHODS

Crassostrea virginica

Oysters were grown in plexiglass trays of 3.3 l volume that were 48.0 cm long, 13.5 cm wide and 5.5 cm deep. The trays were similar to those described by Haven and Morales-Alamo (1970) but were modified to keep the oysters one cm off the bottom. The oysters rested on quarter inch mesh, polyethylene screening which was fitted to the inside dimensions of the tray. The screening was held one cm off the bottom by plexiglass crosspieces glued to the bottom and spaced four cm apart. Oyster feces dropped through the screening and accumulated away from the animals. The trays were operated as open systems. The initial four inches of the tray served as a mixing chamber and was separated by two baffles from the compartment holding the animals. Seawater introduced into the initial chamber was mixed with the test diet by vigorous aeration provided through a one inch airstone. The water flowed down the length of the tray and through a set of baffles before exiting over the end which was one cm lower than the other sides. A dye test indicated that the water passing through the tray remained well mixed and that no static pockets of water existed.

Water from the York River, Va. was used for all experiments. The water was heated to 24.0° C ± 0.5°C by passage through a 13.5 GBR Corning glass heat exchanger. The salinity of the water ranged from 16 °/oo to 21 °/oo . The pH remained between 7.8 and 8.1.

The heated water was filtered through a 10.0 um polypropylene bag filter into a 20 l head box which fed into a trough extending over all growth chambers. The trough was made from a three inch diameter PVC pipe split longitudinally and capped on either end. Water depth in the trough was controlled by adjusting the height of a one inch diameter overflow standpipe. Water from the trough was siphoned into the trays by glass U-tubes 1/4 inch in diameter. The end of each tube immersed in the trough was fitted through a number 4 rubber stopper which was inserted into a hole in a four inch square of plexiglass that rested across the trough. The flow rate into the trays was controlled by adjusting the height of the U-tube in the stopper. The head box, trough, U-tubes and trays were cleaned daily and the bag filters were changed every 12 hours.

Preliminary experiments revealed that substantial quantities of food were passing through the 10 um bag filter. Oysters held in the trays with no supplemental feeding achieved significant growth. To better filter the river water, 12 adult oysters, 3 to 3.5 inches in height, were placed in the head box. These oysters acted as a biological filter and removed any food passing through the bag filter. Oysters held in trays receiving this biologically filtered water with no supplementary diets, grew negligibly.

Diets were metered into the mixing compartments of the trays by a Brinkmann peristaltic pump, model 131900 (Brinkmann Instruments Inc., Westbury, NY). A four liter aspirator bottle served as a reservoir for the diet. Food organisms were added at 3.0 ml/min; the total flow rate of water and food through the tray was 50 ml/min. The reservoirs were

aerated to keep them oxygenated and well mixed. All bottles and lines were cleaned and sterilized daily by autoclaving.

The experimental animals were offspring of a mass spawning (more than 20 adults) of oysters collected from the Wreck Shoals reef of the James River. They were set and grown as cultchless oysters according to the methods of DuPuy et al. (1977). Until used in an experiment, they were held in Nestier trays in flumes receiving raw York River water. At the start of the first experiment, the oysters were three months old and 12 to 20 mm in length.

Each growth experiment had six or seven treatments. For each treatment, 25 oysters ranging from 0.5 to 1.0 g underwater weight were placed in a tray. Their positions in the trays were randomly changed each day to eliminate any effect of position within the tray. Each oyster was wiped clean of slime and labelled with a waterproof felt pen (Sanford Sharpie) prior to the start of an experiment. The animals were individually weighed initially and at the end of a two week period. Growth increments were calculated for each individual and the treatment effects were analyzed by single factor ANOVA. If significant differences (alpha < 0.05) existed among treatments, the results were compared by Student-Neumann-Keuls standardized range test (Zar, 1984).

Six rations of Tetraselmis suecica ranging from 5.0 to 160.0 mg/dry wt/day and a starved control were tested in the first experiment (table IV.1) to determine an optimal ration for comparing the different diets. Based on results of this experiment, all diets were added at a rate of 80 mg dry wt/treatment/day. Each treatment received 0.050 l/min (72 l/day) of filtered estuarine water. The 80.0 mg dry weight of

potential food was metered into each tray over 24 h, resulting in an initial concentration at the head of the tray of 1.11 mg/l.

Monospecific cultures of colorless flagellates, enrichment cultures (described below), T. suecica, and filtered water were compared as oyster foods in experiments two through five (Table IV.2 - IV.5). Monospecific colorless flagellate diets represented three treatments in each experiment. P. vestita, based on initial positive results, was tested in all four experiments. The other two monospecific colorless flagellate diets were varied among experiments, but included two bodonids, a chrysomonad, and a choanoflagellate, described in Section III. Each experiment always included one treatment receiving only filtered water, designated the "starved control". T. suecica and an enrichment culture were likewise included in each experiment.

Six rations of P. vestita ranging from 5.0 to 160 mg dry wgt/day and a starved control were compared in experiment 6 (table IV.6). P. vestita was the only colorless flagellate tested at more than one concentration because it gave the greatest oyster growth of the colorless flagellates examined. In experiments 7, 8, and 9, monospecific diets of T. suecica and P. vestita were compared with diets combining these two species. The six treatments in these experiments consisted of T. suecica (80 mg/day), T. suecica (40 mg/day), P. vestita (80 mg/day), T. suecica and P. vestita (40 mg/day + 40 mg/day), T. suecica and P. vestita (40 mg/day + 80 mg/day), and a starved control. In experiment 10, four species of bacteria, Pseudomonas marina, Aeromonas sp., Flavobacter sp., and Micrococcus sp. were tested along with T. suecica and a starved control. All diets were fed at 80 mg/day.

T. suecica was cultured in 18 l carboys filled with filtered (1.0 um) pasteurized estuarine water enriched with N_2M medium (a mixture of Redfield's solutions A and B), Guillard's vitamin mix, and a manure extract (Dupuy et al., 1977). The T. suecica strain employed (Tetraselmis suecica (Kylin) Butch) was isolated by R. R. L. Guillard at the Woods Hole Oceanographic Institute in 1977 and has been used successfully as an oyster food at the Virginia Institute of Marine Science for over five years. The cultures were not maintained axenic because algae cultured in oyster hatcheries are not usually axenic. The cultures were maintained at 18°C under illumination of 3,000 lux. The salinity of the estuarine culture water ranged from 16 to 21 °/oo. Vigorous bubbling with filtered air kept the cultures well aerated and the cells in suspension. The cultures were harvested in early stationary phase, approximately one week after inoculation. The cell density at harvesting was about 2.0 X 10^6cells/ml.

Colorless flagellates were cultured on a diet of P. marina in 18 l carboys. Filtered (1 um) and pasteurized estuarine water was enriched with BCSF, the nutrient source for the bacteria. The bacteria were inoculated into the carboys 24 h prior to inoculation with flagellates. The carboys were vigorously aerated and maintained at 26°C. Flagellates were harvested when the cultures reached early stationary phase, which occurred in three to four days. The cell density at harvesting ranged from 10^6 to 10^7 cells/l depending on the species being cultured.

Enrichment diets were made by adding BCSF (5g/l) to unfiltered York River water and allowing the microbial flora to bloom. Quantitative counts of microorganisms were not made but microscopic examination revealed that the predominant microorganisms included bacteria,

flagellates, ciliates, amoebae, and fungi. The enrichment cultures were maintained as semi-continuous batch cultures in 36 l carboys. The carboys were harvested daily and refilled with river water enriched with BCSF. New microorganisms were thus continually being introduced into the cultures. The cultures were transfered into clean carboys every week in order to reduce the accumulation of microorganisms growing on the vessel walls. The cultures were vigorously aerated and maintained at 26°C.

The bacteria used in experiment 10 were cultured on a BCSF medium in 18-l carboys, as described for the colorless flagellates. Each carboy was inoculated with 100 ml of bacteria in exponential growth phase in a BCSF medium. Bacterial cultures reached stationary phase in 24 h and were harvested over a four day period without the addition of any fresh media.

Dry weights of microorganisms were determined by filtering three replicate volumes of culture through glass fiber filters (Whatman GF/F, 47mm diameter). Each filter was previously rinsed with 100 ml of particle-free distilled water to remove loose fibers, dried to a constant temperature at 100°C, and weighed. The filters with microorganisms were rinsed with isotonic ammonium formate (0.5 M) to remove sea salts and dried at 100°C for 24 h to volatilize the ammonium formate. The increases in dry weight of the three filters were averaged to yield the dry weight of microorganisms.

Cell counts of the algae and flagellates in culture were made by injecting samples of culture into an A-O "Brightline" hemacytometer and counting the cells at 200X or 450X magnification. Further details on the counting procedure may be found in section III. Since dry weight

determinations required 24 h to complete, the daily rations were calculated from the cell count equvialent of the dry weight ration. No cell counts were made on the enrichment culture; rations of the enrichment cultures were calculated from the last completed dry weight determination. Bacterial rations were similarly based only on dry weight determinations.

Oyster growth was measured by underwater weighing as described by Andrews (1961). Measurements were made with a Sartorius model 1202 MP balance with a digital readout, sensitive to 0.01 g. The balance was supported on a plexiglass frame above a 1-l fingerbowl. An aluminum mesh weighing pan was suspended from the balance into the fingerbowl. Successive weighings were carried out in water of similar depth, salinity, and temperature. Oysters were exposed to the air only during the few seconds required to transfer them from the trays to the finger bowl. Care was taken not to trap air bubbles on or under the shells.

Underwater weighing is an easily performed growth measurement that is both precise and sensitive to the small weight changes associated with juvenile animals. Repeated weighings underwater were precise to 0.01 g. Underwater weight reflects only shell growth, since soft parts have a specific gravity similar to that of seawater. Shell growth was the growth parameter of most interest to the overall objectives of this study. Linear measurements of shell growth were found to be less reproducible due to the irregular growth of the shell margin.

Mercenaria mercenaria

Three experiments were performed to evaluate the growth of juvenile clams on microorganisms cultured on BCSF. The clams were obtained from the VIMS Eastern Shore Laboratory at Wachapreague, Va. (courtesy of M.

Castagna). They were the offspring of a mass spawning of locally collected adults. Upon arrival at Gloucester Point, they were held in trays in flowing York River water for two weeks before use in the first experiment. The mean weight of the clams at the start of these experiments was 0.19 g.

Each experiment involved 16 treatments consisting of five diets tested at three concentrations plus one starved control. The three concentrations were 0.8, 1.6, and 3.2 mg/l. The alga T. suecica was included in all three experiments. In experiment 11 the four other diets were P. vestita, bodonid 1, bodonid 2, and an enrichment culture. In experiment 12 the four other diets were P. vestita, chrysomonad 1, and choanoflagellate 1. In experiment 13 the diets included four bacteria, Pseudomonas marina, Flavobacter sp., Aeromonas sp., and Micrococcus sp. All microorganisms were cultured on BCSF as previously described for C. virginica.

For each treatment ten clams were placed in a beaker containing 4.0 l of filtered (1.0 um), pasteurized, York river water. The salinity of the estuarine water ranged from 18 to 21 ^{o}/oo during the experiments and the pH from 7.8 to 8.2. The beakers were kept in a constant temperature room at 26^{o}C and were gently aerated. Twice a day the beakers were cleaned and the water was changed following which, the microorganisms were added. Clams were observed to start filtering within minutes after the addition of the food.

Each clam was stained black using stamp pad ink 24 h prior to the start of an experiment. Two weeks later at the end of an experiment, the width of the unstained band of new shell growth was measured with an ocular micrometer at 40 X magnification. Growth increments, measured

for each individual in each treatment, were compared with growth of the starved control clams by Dunnett's test for multiple comparisons with a control (Dunnett, 1964). Treatments that were significantly different (alpha = 0.05) from the starved control were then compared by two-way ANOVA which examined the effects of diet and food concentration. The Student-Neumann Keul's standardized range test (alpha = 0.05) was then employed to determine which diets and concentrations differed significantly (Zar, 1984)

RESULTS

Six rations of T. suecica were examined in the first experiment to determine the minimum food required to achieve maximal growth under the experimental conditions employed. Growth increased with increasing ration up to 80.0 mg/day and declined slightly at 160 mg/day (Table IV.1). Pseudofeces did not accumulate in any tray. Although the amount of growth on T. suecica at 40 mg/day was only 81% of that at 80 mg/day, this was not a significant difference ($p<.05$). Growth of oysters fed 5 mg/day was not significantly different from the starved treatments. In the succeeding experiments, all diets were fed at a ration of 80 mg/treatment/day.

Five species of colorless flagellates were evaluated as oyster foods in experiments two through five (Tables IV.2-IV.5). Oyster growth was significantly greater with P. vestita than any other colorless flagellate or starved treatment. Oyster growth on all other species was less than half the growth on P. vestita. Growth on bodonid 1 was significantly greater than the starved treatment in experiments two and three but not in experiment four. This was the only other colorless flagellate diet on which oysters grew significantly more than the starved treatment ($p<.05$). Growth on Bodonid 2, in experiment two, was identical to the starved treatment and this species was not considered further. The choanoflagellate gave poor growth in experiment three but better growth in experiment five. The chrysomonad gave better growth than the choanoflagellate in experiment five and better growth than

Table IV.1

Growth increments over two weeks of juvenile Crassostrea virginica fed sevenconcentrations of T. suecica (n=25), experiment 1.

ration (mg/day)	weight gain + s.d. (g)
0	0.03 + 0.04
5	0.04 + 0.04
10	0.12 + 0.08
20	0.19 + 0.09
40	0.26 + 0.15
80	0.32 + 0.16
160	0.30 + 0.14

One way analysis of variance of the effect of diet upon growth.

Source of variation	Sum of squares	d.f.	Mean square	F ratio	P
between treatments	2.15	6	0.358	29.3	<0.0
within treatments	2.06	168	0.012		

Students-Neumann-Keuls' test for significant differences among diets. Bars underline equal means (alpha = 0.05).

0 mg	5 mg	10 mg	20 mg	40 mg	160 mg	80mg

Table IV.2

Growth increments over two weeks of juvenile *Crassostrea virginica* fed five diets and a starved control (n=25), experiment 2.

diet	mean weight gain + s.d. (g)
P. vestita	0.19 + 0.09
Bodonid 1	0.06 + 0.03
Bodonid 2	0.01 + 0.01
enrichment	0.20 + 0.16
T. suecica	0.28 + 0.16
starved	0.01 + 0.01

One way analysis of variance of the effect of diet upon growth.

Source of variation	Sum of squares	d.f.	Mean square	F ratio	P
between treatments	1.54	5	0.308	41.8	<0.00
within treatments	1.06	144	0.007		

Students-Neumann-Keuls' test for significant differences among diets. Bars underline equal means (alpha = 0.05).

T. suecica	enrichment	*P. vestita*	Bodonid 1	Bodonid 2	starved

Table IV.3

Growth increments over two weeks of juvenile *Crassostrea virginica* fed five diets and a starved control (n=25), experiment 3.

diet	mean weight gain + s.d. (g)
P. vestita	0.22 + 0.10
Bodonid 1	0.08 + 0.03
Choanoflagellate	0.02 + 0.02
enrichment	0.07 + 0.03
T. suecica	0.35 + 0.16
starved	0.01 + 0.01

One way analysis of variance of the effect of diet upon growth.

Source of variation	Sum of squares	d.f.	Mean square	F ratio	P
between treatments	2.21	5	0.442	66.7	<0.0
within treatments	0.95	144	0.006		

Students-Neumann-Keuls' test for significant differences among diets. Bars underline equal means (alpha = 0.05).

T. suecica	*P. vestita*	Bodonid 1	enrichment	Choanoflagellate	starved

Table IV.4

Growth increments over two weeks of juvenile *Crassostrea virginica* fed five diets and a starved control (n=25), experiment 4.

diet	mean weight gain + s.d. (g)
P. yestita	0.15 + 0.08
Chrysomonad	0.06 + 0.04
Bodonid 1	0.03 + 0.02
enrichment	0.09 + 0.06
T. suecica	0.37 + 0.23
starved	0.02 + 0.01

One way analysis of variance of the effect of diet upon growth.

Source of variation	Sum of squares	d.f.	Mean square	F ratio	P
between treatments	2.18	5	0.436	41.4	<0.0
within treatments	1.52	144	0.010		

Students-Neumann-Keuls' test for significant differences among diets. Bars underline equal means (alpha = 0.05).

T. suecica	*P. yestita*	enrichment	Chrysomonad	Bodonid 1	starved

Table IV.5

Growth increments over two weeks of juvenile Crassostrea virginica fed five diets and a starved control (n=25), experiment 5.

diet	mean weight gain + s.d. (g)
P. vestita	0.19 + 0.14
Chrysomonad	0.07 + 0.05
Choanoflagellate	0.06 + 0.03
enrichment	0.22 + 0.15
T. suecica	0.33 + 0.17
starved	0.01 + 0.01

One way analysis of variance of the effect of diet upon growth.

Source of variation	Sum of squares	d.f.	Mean square	F ratio	P
between treatments	1.75	5	0.351	26.8	<0.00
within treatments	1.88	144	0.013		

Students-Neumann-Keuls' test for significant differences among diets. Bars underline equal means (alpha = 0.05).

T. suecica	enrichment	P. vestita	Chrysomonad	Choanoflagellate	starved

bodonid 1 in experiment four, but in neither case was it significantly greater than the starved control. From this limited number of comparisons, the colorless flagellates can be ranked as oyster foods as follows: P. vestita > bodonid 1 > chrysomonad > choanoflagellate > bodonid 2.

The enrichment culture was also examined as an oyster food in experiments two through five. It resulted in greater growth than P. vestita in two experiments (Tables IV.2 and IV.5), but the differences were not significant ($p<.05$). In experiment three it resulted in poorer growth than P. vestita, but was still significantly greater than the starved treatment. In experiment four it resulted in poor growth which was not significantly different from the starved treatment.

Six rations of P. vestita were tested in experiment 6 (Table IV.6). Growth increased with increasing ration up to 80 mg but decreased significantly at 160 mg. There were, however, no significant differences between 20 mg and 40 mg and between 40 mg and 80 mg. Growth on the 5 mg ration was not significantly different from the starved control. Monospecific and combination diets of T. suecica and P. vestita were compared in experiments seven, eight, and nine (Tables IV.7, 8, and 9). T. suecica at 80 mg/day resulted in the greatest growth in all three experiments but only in experiment nine was it significantly greater than all other diets. Diets combining 40 mg/day of T. suecica with 40 mg/day of P. vestita resulted in 86%, 79%, and 71% of the growth that occurred with 80 mg/day of T. suecica. This combination resulted in greater growth than did the diet of 40 mg/day of T. suecica alone, in all three experiments. Only in experiment eight did the combination of 80 mg/day P. vestita with 40 mg/day T. suecica result in more growth

Table IV.6

Growth increments over two weeks of juvenile *Crassostrea virginica* fed seven concentrations of *P. vestita* (n=25), experiment 6.

ration (mg/day)	weight gain + s.d. (g)
0	0.01 + 0.01
5	0.01 + 0.01
10	0.07 + 0.04
20	0.14 + 0.09
40	0.17 + 0.13
80	0.21 + 0.12
160	0.15 + 0.10

One way analysis of variance of the effect of diet upon growth.

Source of variation	Sum of squares	d.f.	Mean square	F ratio	P
between treatments	0.94	6	0.156	21.3	<0.0
within treatments	1.23	168	0.007		

Students-Neumann-Keuls' test for significant differences among diets. Bars underline equal means (alpha = 0.05).

80 mg	40 mg	160 mg	20 mg	10 mg	0 mg	5 mg
———				———	———	
	———	———	———			

Table IV.7

Growth increments over two weeks of juvenile Crassostrea virginica fed algal, protozoal, and combination diets and a starved control (n=25), experiment 7.

diet	composition	ration (mg/day)	mean weight gain + s.d. (g)
1	T. suecica	80.0	0.29 + 0.15
2	T. suecica	40.0	0.21 + 0.11
3	P. vestita	80.0	0.16 + 0.13
4	T. suecica P. vestita	40.0 40.0	0.25 + 0.17
5	T. suecica P. vestita	40.0 80.0	0.24 + 0.15
6	starved		0.01 + 0.01

One way analysis of variance of the effect of diet upon growth.

Source of variation	Sum of squares	d.f.	Mean square	F ratio	P
between treatments	1.25	5	0.249	14.4	<0.00
within treatments	2.49	144	0.017		

Students-Neumann-Keuls' test for significant differences among diets. Bars underline equal means (alpha = 0.05).

diet 1	diet 4	diet 5	diet 2	diet 3	diet 6
———	———				
	———	———	———	———	

Table IV.8

Growth increments over two weeks of juvenile Crassostrea virginica fed algal, protozoal, and combination diets and a starved control (n=25), experiment 8.

diet	composition	ration (mg/day)	mean weight gain + s.d. (g)
1	T. suecica	80.0	0.34 + 0.18
2	T. suecica	40.0	0.22 + 0.13
3	P. vestita	80.0	0.20 + 0.11
4	T. suecica P. vestita	40.0 40.0	0.27 + 0.14
5	T. suecica P. vestita	40.0 80.0	0.29 + 0.15
6	starved		0.01 + 0.01

One way analysis of variance of the effect of diet upon growth.

Source of variation	Sum of squares	d.f.	Mean square	F ratio	P
between treatments	1.66	5	0.333	19.3	<0.00
within treatments	2.48	144	0.017		

Students-Neumann-Keuls' test for significant differences among diets. Bars underline equal means (alpha = 0.05).

diet 1	diet 5	diet 4	diet 3	diet 2	diet 6

Table IV.9

Growth increments over two weeks of juvenile Crassostrea virginica fed algal, protozoal, and combination diets and a starved control (n=25), experiment 9.

diet	composition	ration (mg/day)	mean weight gain + s.d. (g)
1	T. suecica	80.0	0.37 + 0.16
2	T. suecica	40.0	0.24 + 0.14
3	P. vestita	80.0	0.15 + 0.08
4	T. suecica P. vestita	40.0 40.0	0.26 + 0.11
5	T. suecica P. vestita	40.0 80.0	0.23 + 0.13
6	starved		0.01 + 0.01

One way analysis of variance of the effect of diet upon growth.

Source of variation	Sum of squares	d.f.	Mean square	F ratio	P
between treatments	1.86	5	0.373	27.1	<0.00
within treatments	1.99	144	0.013		

Students-Neumann-Keuls' test for significant differences among diets. Bars underline equal means (alpha = 0.05).

diet 1	diet 4	diet 2	diet 5	diet 3	diet 6

than the combination of 40 mg/day P. vestita with 40 mg/day T. suecica. In experiments seven and nine, 80 mg/day of P. vestita resulted in less growth than 40 mg/day of T. suecica, but only in experiment nine was the difference statistically significant.

No growth was noted in any treatment in which oysters were fed a purely bacterial diet (Table IV.10). The mean weight gains of oysters fed bacterial diets were less than those of the starved control oysters. The lack of growth on the bacterial diets was not due to poor water quality since the growth on T. suecica was comparable to that in other experiments. Similarly, poor growth of clams fed bacterial diets occurred in experint 13 (Table IV.14). Only with two bacterial diets, Aeromonas sp. at 3.2 mg/l and Flavobacter sp. at 0.8 mg/l, did clam growth exceed that for the starved control clams and only the former was significantly greater when compared by Dunnett's test (alpha = 0.05). There was no further statistical analysis of this experiment.

Mean oyster growth when fed an 80 mg/day ration of either P. vestita or T. suecica is summarized for all experiments in Table IV.11. Oyster growth on P. vestita is expressed as the percentage of growth that occurred with T. suecica for each of the seven experiments in which they were tested simultaneously. The mean oyster growth on P. vestita was 55% of that on T. suecica. It ranged from 40% in experiment four to 68% in experiment two.

Results of the clam experiments were similar to those of the oyster experiments. The species provided as food had a significant effect upon growth in experiment 11, but food concentration did not (Table IV.12). In experiment 12 (Table IV.13), both species and concentration had significant effects upon growth but a significant interaction occurred

Table IV.10

Growth increments over two weeks of juvenile Crassostrea virginica fed five diets and a starved control (n=25), experiment 10.

diet	mean weight gain + s.d. (g)
Pseudomonas marina	0.007 + 0.007
Aeromonas sp.	0.005 + 0.006
Flavobacter sp.	0.003 + 0.005
Micrococcus sp.	0.002 + 0.004
T. suecica	0.30 + 0.18
starved	0.01 + 0.01

One way analysis of variance of the effect of diet upon growth.

Source of variation	Sum of squares	d.f.	Mean square	F ratio	P
between treatments	1.77	5	0.354	67.3	<0.0
within treatments	0.76	144	0.005		

Students-Neumann-Keuls' test for significant differences among diets. Bars underline equal means (alpha = 0.05).

T. suecica	starved	P. marina	Aeromonas	Flavobacter	Micrococcus

Table IV.11

Comparison of growth of juvenile Crassostrea virginica fed Paraphysomonas vestita and Tetraselmis suecica from nine experiments.

experiment	mean weight gain (grams)		percentage*
	P. vestita	T. suecica	
1	--	0.32	--
2	0.19	0.28	68
3	0.22	0.35	63
4	0.15	0.37	40
5	0.19	0.33	59
6	0.21	--	--
7	0.16	0.29	55
8	0.20	0.34	59
9	0.15	0.37	41
10	--	0.30	--
mean	0.18	0.33	55
standard deviation	0.027	0.033	11
coefficient of variation	0.148	0.10	0.194

* growth on (P. vestita / T. suecica) X 100

Table IV.12

Growth increments over two weeks of juvenile Mercenaria mercenaria fed five diets at three concentrations and a starved control (n=10), experiment 11.

diet	concentration (mg/l)	mean shell increment + s.d. (mm)
P. vestita	0.8	0.40 + 0.17
	1.6	0.42 + 0.23
	3.2	0.59 + 0.20
Bodonid 1	0.8	0.13 + 0.13
	1.6	0.17 + 0.18
	3.2	0.12 + 0.15*
Bodonid 2	0.8	0.20 + 0.18
	1.6	0.19 + 0.19
	3.2	0.10 + 0.08*
T. suecica	0.8	0.59 + 0.19
	1.6	0.81 + 0.31
	3.2	0.59 + 0.30
enrichment	0.8	0.08 + 0.09*
	1.6	0.20 + 0.10
	3.2	0.17 + 0.11
starved		0.07 + 0.10

Two way factorial analysis of variance of the effects of diet and concentration upon growth (starved is not included).

Source of variation	Sum of squares	d.f.	Mean square	F ratio	P
diet	6.92	4	1.73	48.5	<0.00
concentration	0.153	2	0.076	2.14	0.12
interaction	0.541	8	0.068	1.89	0.06
residual	4.81	135	0.036		
total	12.4	149	0.083		

Students-Neumann-Keuls' test for significant differences among diets. Bars underline equal means (alpha = 0.05).

T. suecica P. vestita Bodonid 2 enrichment Bodonid 1

* not significantly different from the starved control (alpha = 0.05; Dunnett's test statistic = 0.036)

Table IV.13

Growth increments over two weeks of juvenile Mercenaria mercenaria fed five diets at three concentrations and a starved control (n=10), experiment 12.

diet	concentration (mg/l)	mean shell increment + s.d. (mm)
P. vestita	0.8	0.24 + 0.11
	1.6	0.38 + 0.24
	3.2	0.52 + 0.17
Chrysomonad	0.8	0.25 + 0.05
	1.6	0.31 + 0.17
	3.2	0.22 + 0.15
Choanoflagellate	0.8	0.05 + 0.06*
	1.6	0.07 + 0.07*
	3.2	0.06 + 0.06*
T. suecica	0.8	0.48 + 0.23
	1.6	0.79 + 0.31
	3.2	0.64 + 0.28
enrichment	0.8	0.18 + 0.15
	1.6	0.18 + 0.17
	3.2	0.10 + 0.09*
starved		0.10 + 0.09

Two way factorial analysis of variance of the effects of diet and concentration upon growth (starved is not included).

Source of variation	Sum of squares	d.f.	Mean square	F ratio	P
diet	3.90	3	0.851	22.8	<0.00
concentration	0.353	2	0.176	4.73	0.01
interaction	0.609	6	0.102	2.73	0.02
residual	4.81	135	0.036		
total	12.4	149	0.083		

Students-Neumann-Keuls' test for significant differences among diets. Bars underline equal means (alpha = 0.05).

T. suecica	P. vestita	Chrysomonad	Enrichment

* not significantly different from the starved control (alpha = 0.05; Dunnett's test statistic = 0.031)

Table IV.14

Growth increments over two weeks of juvenile Mercenaria mercenaria fed five diets at three concentrations and a starved control (n=10), experiment 13.

diet	concentration (mg/l)	mean shell increment + s.d. (mm)
P. marina	0.8	0.05 + 0.07*
	1.6	0.06 + 0.06*
	3.2	0.04 + 0.05*
Aeromonas sp.	0.8	0.05 + 0.06*
	1.6	0.10 + 0.06*
	3.2	0.15 + 0.10
Flavobacter sp.	0.8	0.12 + 0.06*
	1.6	0.03 + 0.05*
	3.2	0.04 + 0.06*
Micrococcus sp.	.8	0.02 + 0.04*
	1.6	0.04 + 0.04*
	3.2	0.03 + 0.05*
T. suecica	0.8	0.62 + 0.18
	1.6	0.79 + 0.33
	3.2	0.60 + 0.31
starved		0.11 + 0.08

* not significantly different from the starved control (alpha = 0.05; Dunnett's test statistic = 0.019)

between them. For this reason, the diets were compared by the Student-Neumann-Keul's test across all concentrations. In both experiments growth was best for a T. *suecica* diet and significantly greater than for any other diet. P. *yestita* was again the best colorless flagellate diet and significantly greater growth occurred on P. *yestita* than on the other colorless flagellate species in both experiments. When compared across all concentrations, growth on P. *yestita* was 71% and 59% respectively, of the growth on T. *suecica*. In experiment 11, both bodonid 1 and bodonid 2 had treatments that did not differ significantly from the starved control. In experiment 12, growth on the choanoflagellate at all concentrations was less than that for the starved control. Growth of clams fed the chrysomonad at all concentrations was significantly greater than the starved control clams but was still significantly less than growth of clams fed P. *yestita*. Growth on the enrichment cultures was poor in both experiments.

DISCUSSION

Bivalve growth is highly dependent on water quality. Short term fluctuations in water quality often make the results of sequential growth experiments difficult to interpret. This is especially true when experiments are performed without any physio-chemical treatment of the water supply, as in this study. Subtle yet adverse changes in water quality may result from either human activities or natural causes such as blooms of marine organisms. Such changes in water quality may account for the great variability often obtained in bivalve rearing studies (Walne, 1970; Kern, 1974). To facilitate comparisons of different experiments, T. suecica was used as a treatment against which all diets could be compared. Poor growth of oysters in all treatments including those fed T. suecica, could be an indication of poor water quality during that particular experiment. T. suecica was used because it has been repeatedly demonstrated to be a good food for bivalve larvae (Davis and Guillard, 1959; Loosanoff and Davis, 1963) and juveniles (Cole, 1937; Walne, 1970; Helm, 1977; Langdon and Waldock, 1981; Creekman, 1977; Wikfors et al., 1984). Webb and Chu (1983) rated T. suecica first out of nine algae in terms of protein quality for bivalves. In a survey of shellfish rearing facilities in N. America and Europe, T. suecica was the third most widely used algal species for rearing bivalves (Cost, 1978 cited in Persoone and Claus, 1980). T. suecica has been used successfully at VIMS for about 10 years to condition C. virginica for spawning.

Maximum growth of oysters in this study was obtained with a ration of 80 mg dry wt/treatment/day, equivalent to 3.2 mg dry wt/oyster/day. Epifanio and Ewart (1977) derived an equation to predict the maximum daily ration of oysters based on the relationship between size and rate of filtration. Their equation predicted that the maximum daily ration for an oyster of the size used in this study (approximately 1.5 g whole weight) would be 12.7 mg dry wt/day, a four fold greater ration than that used. Maximum daily ration, however, is not synonymous with the ration that permits maximum growth. In addition, as they stated in their discussion, their equation may overestimate the ration for small oysters. A ration of T. suecica of 80 mg/day is equivalent to a cellular concentration at the head of the tray of approximately 16 X 10^3 cells/ml. This value falls within the range of concentrations of T. suecica (10 to 30 X 10^3cells/ml) which Walne (1970) found to yield the best growth of juvenile Ostrea edulis. Three concentrations of food (0.8 mg/l, 1.6 mg/l, and 3.2 mg/l) were tested in the clam experiments to see if food were limiting and if growth would increase with increasing concentration. No obvious overall relationships between concentration and growth were observed. In only one treatment, P. vestita in experiment 12, did growth improve markedly with increasing concentration. The two-way ANOVA indicated that concentration did not significantly affect growth in experiment 11. Concentration did have a significant effect on growth in experiment 12 but a significant interaction between diet and concentration also occurred. A diet with little nutritional value would not be expected to affect growth at any concentration, whereas one of high nutritional value would. No conclusions about the effects of concentration can be drawn from these

limited experiments because of the small sample size of the treatments (n=10) and the large inherent variability in growth of individual clams.

Significant growth was observed on only two of the five flagellate species examined as oyster foods, P. vestita and bodonid 1. Of these two, P. vestita was by far the better food, yet oysters fed P. vestita averaged only 55% of the growth observed with T. suecica. For clams, the growth on P. vestita was 71% and 59% of that on T. suecica. Four factors have been suggested to explain why some algal species are better bivalve foods than others: cell size, toxicity, possession of a digestible cell wall, and nutritional value (Ukeles, 1969). These factors, which are not mutually exclusive, would similarly apply to colorless flagellates.

The sizes of the colorless flagellates studied range from approximately 2.0 to 8.0 um in diameter. This is within the range of particle sizes filtered from suspension by oysters (Haven and Morales-Alamo, 1970) and clams (Rice and Smith, 1958), although the smaller flagellate species are of a size that is filtered from suspension with less efficiency. P. vestita (8 um), the best food, was the largest of the flagellates. However, algal cells such as Nannochloris and Chlorella, which are as small as 1 to 2 um, have been successfully used as oyster foods (Dupuy, 1975).

Colorless flagellates may excrete extracellular products or metabolites which are toxic to bivalves but no published studies have examined this point. Toxin producing algae have been well documented and are found in the algal classes Cyanophyceae, Dinophyceae, Chlorophyceae, and Prymnesiophyceae (Carmichael et al., 1985; Shilo, 1971). The latter two families contain several species which are

excellent foods for bivalves (Walne, 1970). Guillard (1958) examined 37 algal isolates in six classes and found varying degrees of toxicity in the species Amphidinium, Gymnodinium, Chlorella Stichococcus, and Prymnesium. A consideration in culturing colorless flagellates is ensuring that the bacteria on which they graze are not pathogenic to bivalves or toxin producing. Although the bacteria in the flagellate cultures were grazed to low concentrations, generally less than 10^5 cells/ml, they were not eliminated from the cultures. Guillard (1958) found that bacterial contaminants in algal cultures could make good foods toxic. In this study, all colorless flagellate species were raised on P. marina, which has not been implicated as a bivalve pathogen. It is unlikely that it produces toxic by-products since this would have produced a deleterious effect on all treatments with flagellate species.

Foods for both larvae and adults may be poor as a result of the inability of the bivalve's digestive system to break down the cell wall of the food organism. Davis and Guillard (1958) discovered that young oyster and clam larvae grew better on naked phytoflagellates then on species with thick cell walls. Walne (1974) suggested that poor growth of Ostrea edulis larvae fed on Chlorella and Coccomyxa was due to thick cell walls, which could not be digested. Babinchek and Ukeles (1979), using fluorescence microscopy, observed that Chlorella autotrophica cells were not lysed in the gut of C. virginica larvae; the cell walls appeared to be resistant to the larval digestive enzymes. Dean (1957) observed that some algal species disintegrated immediately upon contact with an extract of the crystalline style of an oyster while other species did not. He suggested this as a quick method of evaluating the

food potential of different species. Epifanio (1982) attributed the poor growth of adult C. virginica on Phaeodactylum tricornutum to the alga being relatively indigestable rather than to its gross composition or lack of a micronutrient. Bricelj et al. (1984) found that poorly digested algal species pass through the gut of M. mercenaria more quickly than species that are well digested. The poor digestion of certain species of Chlorophytes was attributed to the presence of a highly refractory trilaminarin cell wall component that is resistant to enzymatic attack.

It is unlikely that poor growth of oysters on some colorless flagellate species in this study resulted from an inability to digest the cell wall. All species tested lacked the thickened cellulose cell walls characteristic of those algal species that are poorly digested. Flagellates dependent on phagotrophic nutrition may require a certain plasticity and freedom of movement to aid in prey capture. This would preclude development of rigid body forms. Bodonids have a relatively thin cell membrane without any scales or plates that covers an inner pellicle composed of microtubules (Brooker, 1971; Vickerman and Preston, 1976). A related species, Leptomonas collosoma, has a cell membrane that is composed of 52% protein, 29% lipid, and 8% carbohydrate (Cook and Stoddard, 1973). Chrysomonads, such as P. vestita and the unidentified Chrysomonad, have a naked protoplast bounded by a plasma membrane and covered with silica scales (Dodge, 1973; Farmer, 1980). The lack of an underlying rigid pellicle or cell wall is evidenced by observing their extreme plasticity when feeding. P. vestita is characterized by its elongated, highly ornate spines (Wee, 1982), which apparently do not interfere with its being ingested by bivalves.

Choanoflagellates may have a lorica of cellulose or chitin but the species used in this study was a member of the family Codosigidae (section III), a naked form that lacks a lorica (Sieburth, 1979). The lorica, regardless of its composition should not interfere with the digestion of the cell since it does not completely encase the cell, which would still be exposed to the action of digestive enzymes. Like the other flagellates tested, the choanoflagellate also exhibited a high degree of plasticity.

The value of a bivalve food is difficult to determine because of a lack of understanding of the nutritional requirements of bivalves. Requirements for protein, carbohydrate, and lipid or requirements for specific micronutrients and vitamins have yet to be determined for either M. mercenaria or C. virginica (Epifanio, 1983; Langdon, 1983; Webb and Chu, 1983). Any interpretation of growth studies based on composition of organisms in the diet is therefore highly speculative.

P. vestita, the colorless flagellate on which the best shellfish growth was obtained, maintains its energy stores in the form of leucosin and oil (Sieburth, 1979). Leucosin, also known as chrysolaminarin, is composed of 1,3 glucose residues which can be hydrolyzed by the digestive enzymes found in bivalves (Reid, 1983). The lipid content of P. vestita has never been analyzed, but generally, one third of the dry weight of a chrysophyte is lipid (Aaronson, 1980). In addition, they tend to be rich in long chain polyunsaturated fatty acids which may be necessary for bivalve growth (Webb and Chu, 1983). The poor growth obtained on the bodonids may be related to a lack of carbohydrate reserves, a characteristic of kinetoplastid flagellates (Vickerman and Preston, 1976). Their lipid content may be very minimal as well; the

only energy stores may be in the form of protein (Vickerman and Preston, 1976). Choanoflagellates have food stores in the form of lipid (Sleigh, 1973) but little is known of the percent composition or types of fatty acids present. There are no reports of polysaccharide food reserves in choanoflagellates.

The composition and nutritional value of P. vestita to bivalves may be affected by the conditions under which it was cultured. It has been demonstrated that the formulation of an algal culture medium may affect the nutritional value of an alga (Wikfors et al., 1984). The bacterial strain upon which a colorless flagellate is cultured, may similarly affect the nutritional value of the flagellate. In addition, the conditions under which the bacteria were cultured may be reflected in the flagellate's composition. The phase of growth (Flaak and Epifanio, 1978) and population growth rate (Gallagher and Mann, 1981) affect the composition and nutritional value of an alga. The composition and size of colorless flagellates may be similarly affected by food availability (Fenchel, 1982). Colorless flagellates in early stationary phase, as used in this study, may have a very different nutritional value to shellfish than that of exponential or late stationary phase cultures.

Oyster growth on monospecific and combination diets of P. vestita and T. suecica were compared in experiments 7, 8, and 9 (tables IV.7, 8, and 9). No combination diet was able to produce greater growth than a pure diet of T. suecica. An 80 mg ration that was 50% P. vestita did not produce the same growth as a 100% T. suecica diet. This suggests that the reduced growth on P. vestita is not simply a lack of a limiting trace nutrient that could be supplied by the algae. P. vestita may have less caloric value than an equal weight of T. suecica or it could have

the same value but be less completely assimilated and digested. A similar situation was encountered by Epifanio (1983) in trying to explain the poor nutritional value of Phaeodactylum tricornutum to oysters. P. tricornutum had little nutritional value even when combined with Thalassiosira pseudonana, a good food. The differences in food value could not be explained by gross composition, fatty acids or amino acids of the diets. Studies comparing assimilation efficiency of the two algae by oysters suggested that P. tricornutum was poorly digested.

In experiments 7 and 9 a combined ration of 40 mg of T. suecica with 80 mg of P. vestita gave less growth than a ration with 40 mg of P. vestita. The presence of an inhibitory product in the P. vestita culture could cause this result. Another explanation is that when high concentrations of a poorly digested food are present, the amount of easily digested food ingested is reduced. When 80 mg of P. vestita are combined with 40 mg of T. suecica, less of the T. suecica is ingested than when only 40 mg of P. vestita are present. Increasing the ration with a poor food beyond the level that saturates the rate of ingestion will only dilute the amount of the better food ingested. When the ration is low and ingestion is not limiting, any additional food, even if it is of poor quality, will still be ingested without reducing the amount of the good food ingested. This could explain why 40 mg of P. vestita with 40 mg of T. suecica would still yield better growth than 40 mg of T. suecica alone. Ingestion is not limiting at this lower ration and no dilution of the T. suecica occurs.

Numerous reports on the use of colorless flagellates as food for bivalves are found in the Japanese scientific literature, but most of these studies deal with larval bivalves and not with juveniles or

adults. Imai and Hatanaka (1949) raised larvae of C. gigas in small stationary cultures on a colorless flagellate they identified as Monas sp. The genus Monas, a name widely used in the older literature, is believed to be synonomous with the genus Paraphysomonas (Sieburth, 1979). The flagellates in these studies were cultured in a seawater-hay infusion. Imai et al. (1950) reported the successful use of Monas in large scale cultivation of C. gigas in outdoor tanks. The tanks, ranging from 2,500 l to 19,000 l capacity, were fertilized with starch and attained concentrations of cells exceeding 10^3 cells/ml. Larvae raised in these tanks had growth rates comparable to those in nature. A limited number of experiments examined the growth of larvae of C. virginica, which did successfully metamorphosize on a Monas diet, but did not grow or survive as well as the C. gigas larvae. This difference was attributed to the poor condition of the broodstock and not to the diet. Larvae of Ostrea edulis (Imai et al., 1953) and Ostrea lurida (Imai et al., 1954) were successfully raised on a diet of Monas through metamorphosis and up to an age of several months at which time they were planted in bays. In these studies the tanks were fertilized with cooked soluble starch or cane sugar to provide bacteria for the flagellates. Other bivalve larvae successfully cultured on a Monas diet included Mactra sachalinensis (Imai et al., 1954), Anadara broughtonii (Kan-no and Kikuchi, 1962), Meretrix meretrix, Venerupis phillipinarum, Chlamys farreri, and Teredo navalis (cited in Imai, 1977). Wada (1973) compared the growth of larvae of Pinctata fucata on diets of Chaetoceos, Chlorella, and Monochrysis to growth on Monas (Kobayashi and Yuki, 1952) and concluded that growth on Chaetoceros, the best algal diet, was similar to growth on Monas.

Colorless flagellates, recently recognized as an important component of plankton biomass (Davis, 1982), may represent an important source of nutrients for bivalves in nature. It would however, be difficult to quantify their contribution to shellfish production. Because of their delicate structure, they would easily disintegrate in a bivalve stomach. For this reason they have probably been overlooked in the many early studies that analyzed gut contents of bivalves (reviewed in Galtsoff, 1964). Imai et al. (1950), in an ecological study of Japanese bays, concluded that blooms of colorless flagellates, resulting from decomposition of eel-grass, were responsible for successful seed-oyster production. Oyster spawning was correlated with peaks in the numbers of colorless flagellates which at times exceeded 10^4 cells/ml.

Outside of the japanese scientific literature, there are few references to use of colorless flagellates for rearing bivalves, all of them negative. Davis (1950) reported that C. virginica larvae were unable to grow on the colorless euglenoid Astasia klebsii or on a colorless monad. Walne (1956) evaluated the use of Bodo sp. as a food for O. edulis larvae and found that larvae were unable to grow on it. In their monograph on larval culture Loosanoff and Davis (1963) state that they were unable to culture larvae of C. virginica or M. mercenaria on the same strain of Monas sp. that was used by Imai in Japan. They did not explain how they cultured the Monas sp. and their poor results may be due to the bacterial species used to culture the flagellates. In the early literature there are numerous references to the importance of protozoa or infusoria in the diets of oysters (reviewed in Martin, 1928), but most of these studies were based on gut examinations and were not controlled feeding experiments.

No growth was observed on bacterial diets for either oysters (table IV.10) or clams (table IV.4). Since there was no growth of bivalves fed bacteria, this diet was not tested after the initial experiments with each bivalve. The animals in both experiments were observed to actively feed and no mortality occurred during the experiments. This suggests that bacterial extracellular products or metabolites were not inhibiting the animals. Many suspension feeding bivalves are capable of filtering bacteria from suspension (McHenery and Birkbeck, 1985) and ingestion of bacteria by oysters and clams has been well documented (Cabelli and Hefferman, 1970; Perkins et al., 1980). Both oysters and clams are capable of filtering particles in the bacterial size range but the efficiency of particle retention by the gills may be much lower than for large particles (Haven and Morales-Alamo, 1970; Rice and Smith, 1958).

Few studies have demonstrated growth of bivalves on a defined bacterial diet under controlled conditions. Zobell and Landon (1937) showed that Mytilus californianus was able to increase in body weight when fed an exclusively bacterial diet over a nine month period. Hidu and Tubiash (1963) used antibiotics to select for growth of resistant bacteria which resulted in improved growth of larval oysters and clams, but the species of bacteria involved were not isolated or identified. Bivalves living adjacent to hydrothermal vents are able to grow on hydrogen sulfide oxidizing bacteria (Corliss et al., 1979).

Not all bacteria may be capable of supporting bivalve growth. Davis (1950, 1953) fed thirteen strains of marine bacteria to larval oysters and found that none of them would support growth. Adult C. virginica were unable to grow on Vibrio angullarium, Escherichia coli, or Aeromonas salmonicida (Tubiash, 1974). These species are poor

choices as potential foods since they are all known pathogens for other organisms.

The enrichment culture provided significantly greater oyster growth than the starved control in three out of the four experiments in which it was tested (tables IV.2-5) and in two of those experiments (tables IV.2 and IV.5) it was a better diet then the colorless flagellates tested. The enrichment was a poor source of nutrition for clams in both experiments (tables IV.12 and IV.13) in which it was tested. It provided a good diet for oysters, at times, but was not consistently good. This lack of consistency is a serious drawback for its use in mariculture. A food culture must be dependable in order to achieve any acceptance in the mariculture industry. The different nutritional values of enrichment cultures may reflect a change in their species composition. The predominant microorganisms included bacteria, fungi, ciliates, colorless flagellates, and amoebae, but no quantifications or identifications of these microorganisms was attempted. It is unknown which of these organisms or which combinations of organisms were providing nutrition for the bivalves. Isolating the major microbial constituents from an enrichment culture that resulted in good nutrition might lead to discovery of potentially valuable heterotrophic food organisms

Many attempts have been made to culture bivalves on organic compounds of plant, animal, and microbial origin. These compounds have been tested in particulate, colloidal, and soluble forms. The addition of these compounds to non-sterile seawater results in a bloom of heterotrophic microorganisms, similar to an enrichment culture. In some of these studies the water was filtered to remove large microorganisms

but rarely were bacteria excluded. Colorless flagellates as well, may have been present in these diets. Doubling times on the order of 20 minutes are possible for bacteria and four hours for colorless flagellates (Section III). At these rapid division rates, a warm, well aerated food reservoir may rapidly bloom with heterotrophic microorganisms. When such cultures are used to raise bivalves it is difficult to separate growth effects of the original compounds from those of the "bloomed" microorganisms. Like the enrichment culture in this study, such diets are often inconsistent in food value, possibly because of changes in microbial composition.

Nelson (1934) in an early attempt to raise oysters on organic compounds, evaluated the corn starch, ground alfalfa, soya bean meal, and ground crab meat, of which only corn starch resulted in any growth. Carriker (1961) used Pablum cereal to feed clam larvae but experienced high mortality which he attributed to growth of microorganisms. Boullion, nutrient agar, lactose broth, milk, barley extract, and carbohydrate and amino acid solutions were among the many organic compounds tested by Chanley and Normandin (1967) as foods for larval clams, none of which resulted in any growth. They did achieve some success with finely ground macrophytic algae. Very similar results were obtained with M. galloprovincialis larvae (Masson, 1977) which grew poorly with dissolved or colloidal compounds but did grow on ground macrophytes, and achieved best growth with lyophilized phytoplankton. Dissolved organic compounds may in general, stimulate quicker bacterial growth than particulates, and this may be responsible for much of the extremely poor growth and high mortality observed with dissolved diets.

Feeding of cereal flours, rich in carbohydrates, results in consistent increases in oyster dry tissue weights (Haven, 1965; Kuwatani and Nishii, 1968; Dunathan et al., 1969). In the case of corn starch, the role of microorganisms can be eliminated since the food cultures were maintained sterile (Haven, 1965; Turgeon and Haven, 1978). An additional problem in interpreting feeding studies is that the physiological state of an animal, which is a function of its environmental history, may influence how an animal responds to a particular diet. Turgeon and Haven (1978) demonstrated that carbohydrate utilization in oysters is significantly affected by season because of its relation to the spawning cycle. A similar finding for clams was reported by Harleston (1971).

The most promising approach to using BCS for bivalve mariculture is to culture bacteria on the BCS, raise the colorless flagellate P. vestita on the bacteria, and then feed the flagellate to bivalves. The flagellate link in the food chain is necessary for proper bivalve nutrition given the total lack of growth on bacterial diets. Enrichment cultures, as previously described, are not capable of providing consistently good nutrition, a serious drawback to their use.

This study demonstrated that juvenile bivalves are capable of growth on P. vestita but do not grow as well as on T. suecica. The nutritional value of this colorless flagellate was compared to a strain of algae that was chosen on the basis of over fifty years of research and dozens of investigations on algal diets. Of the hundreds of strains of colorless flagellates that may exist, only five were evaluated in this study. Among these other untested strains, most of which have yet

to be cultured, there may be organisms which are superior to P. vestita in nutritional value and are the equal of any algal diet.

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SECTION V

Conclusions

The potential for use of BCS in bivalve culture was assessed by comparing the growth of juvenile oysters fed colorless flagellates with their growth on algae. Growth rates and efficiences for juvenile oysters under simulated hatchery conditions can be estimated from the results in section IV. The requirements for culturing food organisms (bacteria and protozoa) for use in an oyster hatchery using BCS as a nutrient source can be calculated from the results of sections II and III. These calculations were made for a small hatchery capable of producing daily 15,000 juvenile oysters weighing 2.0 g each, for transfer to natural beds (Table V.1). The requirements of a Tetraselmis based hatchery capable of the same production are presented in Table V.2. Numerous simplifying assumptions (i.e. absence of mortality, constant growth rate) were made in the following discussion which is not intended to be a detailed production model of a bivalve hatchery.

Growth rates of oysters in a hatchery are estimated from the results presented in table IV.11. The best growth of oysters fed I. suecica was 0.37 g underwater weight over a 14 day experiment, which was equivalent to a daily growth increment of 26.4 mg underwater weight/day. Since underwater weight is approximately 50% of the whole weight, the growth rate of these oysters expressed as whole weight is estimated to have been 52.8 mg/day. The greatest growth of oysters fed P. vestita

Table V.1

Oyster Production and Culture Requirements for an Oyster Hatchery Using BCS to Raise Bacteria and Protozoa.

daily hatchery production	15,000 2.0-g oysters
oyster specific growth rate	0.021 day^{-1}
time required to reach 2.0 g	180 days
biomass of oysters	1,575 kg
daily protozoan production	2.98 kg
protozoan culture volume	2,811 l
daily bacterial production	7.8 kg
bacterial culture volume	784 l
daily BCS requirement	61.8 kg

Table V.2

Oyster production and culture requirements for an oyster hatchery using Tetraselmis suecica.

daily hatchery production	15,000 2.0-g oysters
oyster specific growth rate	0.035 day^{-1}
time required to reach 2.0 g	106 days
biomass of oysters	1,105 kg
daily algal production	1.77 kg
algal culture volume	6,156 l
culture vessels	77 80-l vessels

was 0.22 g underwater weight over a 14 day experiment which is equivalent to a daily growth increment of 31.4 mg/day, when expressed as whole weight.

Growth efficiency was considered to be the daily growth increment divided by the daily ration. The daily ration was 80 mg dry weight of algae or protozoa per treatment of 25 oysters which was equivalent to 3.2 mg dry weight/oyster/day. For oysters fed T. suecica the growth efficiency was 16.5 mg-oyster/ mg dry weight of algae and for those fed a P. vestita diet, 9.81 mg-oyster/ mg dry weight of protozoan. The growth efficiency on P. vestita was 60% of that on algae. The growth efficiencies were greater than 1.0 because the oyster weight is whole weight (or wet weight with shell) while the algae were expressed as dry weight. The dry meat weight of oysters this size is approximately 3% of their whole weight (Muller, 1978). Using this conversion factor, the growth efficiencies expressed as mg- dry meat weight per g dry weight of food are 495 for growth on T. suecica and 294 for growth on P. vestita.

The overall growth efficiency of oysters on BCS was equal to the product of the yield of bacteria (P. marina) on BCS, the yield of protozoa (P. vestita) on bacteria, and the growth efficiency of oysters on protozoa (Table V.3). The yield of P. marina on BCS was 127 mg (dry weight) per g (dry weight) of BCS (Table II.8a). The yield of P. vestita was 380 mg (dry weight) per g (dry weight) of P. marina (Table III.6a). The growth efficiency of oysters on P. vestita, as previously calculated, was 9,810 mg oyster (whole weight) per g (dry weight) of P. vestita or 294 mg oyster (dry meat) per g of P. vestita. The overall growth efficiency of oysters on BCS was thus 473 mg of oyster (whole

Table V.3

Overall Conversion Efficiency of a BCS Based Aquaculture System

trophic stage	transfer efficiency mg/g
BCS to bacteria	127
bacteria to protozoa	380
protozoa to oyster (whole weight)	9,810
protozoa to oyster (dry meat weight)	294
BCS to oyster (whole weight)	473
BCS to oyster (dry meat weight)	14.2

weight) per g of BCS (dry weight) or 14.2 mg of oyster (dry meat) per g of BCS (dry weight).

Only 1.4% of the BCS is converted to oyster biomass if the overall growth efficiency of the oysters is expressed as dry meat weight (Table V.3). Likewise, a similarly small percentage of the nutrients in the BCS must be converted to oyster biomass. The reason for the low overall efficiency is that the nutrients are passing through three trophic levels in the aquaculture scheme with metabolic losses occurring at each step. In addition, the first transfer from BCS to bacteria is particularly low because the bacteria are not utilizing all the compounds in the BCS. Nutrients not converted to oyster biomass are being released to the environment as metabolic products or undigested biomass. Since only a small percentage of the nutrients are recycled, this scheme is an inefficient way to recycle nutrients. As a form of waste treatment, this wastewater aquaculture scheme has no advantages over conventional forms of waste treatment in removal of nutrients. It may still have economic advantages to the operation of a bivalve hatchery.

Juvenile oysters in a hatchery can be considered to grow exponentially and specific growth rates (k) are therefore calculated using such a model. The growth of young rapidly growing organisms can be described by an exponential model (Allen et al., 1984). Values of k can then be calculated from the following formula:

$$k = \frac{1}{t} \ln \frac{Wt}{Wo}$$

where, k = specific growth rate, $days^{-1}$

t = growth period, days

Wo= average initial oyster weight, g

Wt= average final oyster weight, g

The specific growth rate was determined by considering the growth period (t) to be 1.0 day and the final oyster weight (Wt) to be the initial weight (Wo) + the daily growth increment. The specific growth rate of oysters fed P. vestita was 0.0205 day^{-1}, which is equivalent to 2.1% day^{-1}, and the specific growth rate on T. suecica was 0.0346 day^{-1} or 3.5% day^{-1}. These values, which were based on oysters ranging from 1.5 g to 2.0 g (whole weight) may be underestimates of the specific growth rate of small oysters. For this discussion, however, the specific growth rates will be considered to remain constant with increasing size, up to 2.0 g.

The exponential growth model can be used to calculate the amount of time (t) required for a newly set oyster (Wo) to reach 2.0 g (Wt), the size at which it leaves the hatchery. Rearranging the previous equation we obtain:

$$t = \frac{1}{k} \ln \frac{Wt}{Wo}$$

In this case Wt is considered to be 2.0 g and Wo, the weight of a newly set spat, is considered to be 0.05 g. For growth on P. vestita, with k = 0.0205 day^{-1}, 180 days are required to reach 2.0 g and for growth on T. suecica, with k = 0.0346 day^{-1}, 106 days are required.

If a hatchery is to continually produce juveniles which are removed when they reach a given size (i.e. 2.0 g), it must maintain a stable population structure. The number of animals in each age class (in this example, day class) must remain constant. The hatchery biomass required

to produce a single 2.0 g oyster on a daily basis was approximated by a geometric series:

$$S = Wo\left[\frac{1-(g)^{t+1}}{1-q}-\right)$$

where,

S - hatchery biomass required to produce a single 2.0 g oyster on a daily basis

Wo= initial weight, 0.05 g

t = days required to reach 2.0 g

q = ratio of oyster weights on successive days

The total oyster hatchery biomass required to produce 15,000 2.0 g oysters on a daily basis is calculated to be (15,000)(S) or 1,575 kg for growth on P. vestita, with a 180 day growing period, and was 1,105 kg for growth on T. suecica with a 106 day growing period.

Mortality of spat was not taken into account in making these biomass estimates. In a real hatchery, mortality would have a significant effect. If we assume that oysters fed algal and protozoan diets have the same mortality rate, the diet producing slower growth will result in greater mortality due to the longer period of time the animals remain in the hatchery. In order to end up with the same number of animals, a larger initial biomass will be required for the slower growing diet. Mortality effects can be described by exponential decay equations (Everheart et al., 1975). Assuming a constant mortality rate of 1% (0.01 day^{-1}), a hatchery requiring a 180 day growing period (P.

galbana diet) must start out with 90,909 spat to end up with 15,000 oysters. A hatchery with a 106 day growing period (*T. suecica* diet) need start out with only 43,353 spat to end up with 15,000 oysters. A detailed discussion on the effects of mortality on the operation of a hatchery is beyond the scope of this discussion and mortality effects will not be considered further.

The total amount of oyster growth that occurs each day can be estimated from the growth increment of each daily age class multiplied by the number of oysters in each age class. Since mortality is assumed to be zero, the number of oysters in each age class remains constant at 15,000. The sum of the growth increments for each age class is equal to the final weight minus the initial weight or 2.0 g - 0.05 g = 1.95 g. The total amount of growth is (15,000)(1.95g) = 25,250 g or 25.25 kg. In order to harvest 15,000 2.0 g oysters equivalent to 30,000 g of oyster biomass daily, the hatchery stock must increase in weight by 25.25 kg. An additional 0.75 kg will be contributed by newly set animals.

The daily algal production required for this hatchery can be estimated as the total amount of oyster growth (29.25 kg) divided by the efficiency of oyster growth on *T. suecica* (16.5 kg of oyster per kg dry weight of algae). The result, 1.77 kg (dry weight) of *T. suecica*, is the amount of algae that must be produced daily by the hatchery.

Large scale culture of *T. suecica* for use as an oyster food has been studied by Laing and Jones (1983). They found that under optimal conditions, an 80 l culture vessel, with a cell density of 2×10^6 cells/ml, could produce 23 g (dry weight) of algae per day. To produce the 1.77 kg of algae required by the theoretical hatchery under

discussion, would require 77 culture vessels of 80 l or a total of 6,156 l.

The daily production of P. vestita required for this theoretical hatchery can be estimated from the total amount of oyster growth (29.25 kg) divided by the efficiency of oyster growth on P. vestita (9.81 kg of oyster per kg dry weight of P. vestita). The result, 2.98 kg (dry weight) of P. vestita, is the amount that must be produced daily by the hatchery.

The volume of culture required to produce 2.98 kg (dry weight) of P. vestita is calculated from the specific growth rates and cell densities measured in section III, using the following equation for exponential growth:

$$N_t = N_0 e^{kt}$$

where,

N_t = final cell concentration

N_o = initial cell concentration

k = specific growth rate, h^{-1}

t = amount of time in growth, days

e = base of natural logarithms

A culture with an initial cell density of 10^6 cells/ml and in exponential growth for 24 h with a specific growth rate of 0.105 h^{-1} (table III.6b) would have a final cell concentration of 12.4 X 10^6 cells/ml. The daily production of such a culture is calculated as the final concentration minus the initial concentration, equivalent to 11.4 X 10^6 cells/ml. The daily production in units of g (dry weight)/l is 1.06 g (dry weight)/l, which can be obtained by multiplying the dry weight of a single P.

vestita cell (9.3×10^{-11} g/cell) and converting from ml to l. Dividing the required amount of P. vestita (2.98 kg) by the dry weight production (1.06 g/l) yields 2,811 l, the required volume of P. vestita culture. If P. vestita were to be produced in 80 l culture vessels, 35 vessels would be required.

P. vestita, to be fed to oysters, requires bacteria (P. marina) for nutrition. The amount of bacterial biomass required daily can be estimated by dividing the required biomass of P. vestita (2.98 kg) by the yield of P. vestita on P. marina (0.38), or 7.84 kg of P. marina. The required volume of bacterial culture, however, would be much lower than the flagellate culture volume because the bacteria have a much higher specific growth rate. The specific growth rate for P. marina was 0.411 h^{-1} (table 11.8), which is equivalent to an approximately 20,000 fold increase when growing exponentially for 24 h. If the P. marina is harvested daily from a batch culture when the concentration reaches 10 g/l, a culture volume of 784 l would be required. This culture would require a daily inoculation of only 0.41 g of P. marina.

The amount of BCS required daily for the bivalve hatchery under discussion is 61.8 kg, which was estimated by dividing the daily increase in oyster biomass (29.25 kg) by the overall yield of oysters on BCS (0.473). The same value for BCS would also be obtained by dividing the required amount of P. marina by the yield of P. marina on BCS. Considering that a large modern brewery such as the Anheuser Busch brewery in Williamsburg, produces on the order of 14 million liters of waste water per day, a single oyster hatchery using 61.8 kg of BCS per day is using a negligible fraction of the waste effluent.

A bivalve hatchery using BCS as a nutrient source would require considerably less space for culturing the food organisms for bivalves than would a comparable hatchery raising algae. The daily production of 15,000 2.0 g oysters would require a 2,811 l culture for P. yeatita and a 784 l culture for P. marina, or a total culture volume of 3,595 l. A bivalve hatchery with the same daily production and using T. suecica for food would require 6,156 l for algal production. The BCS based hatchery requires only 58% of the culture volume that the algal based hatchery requires. In addition, the cultures for the BCS based hatchery can utilize floor space more efficiently than algal cultures. Algal production is based on the use of 80 l culture vessels and the required 77 vessels would cover 6.93 square meters. The algal cultures can not be easily scaled up to larger volumes because of the limitations imposed by light penetration. The 2,811 l of P. yeatita culture could be raised in a single culture vessel. A cylindrical tank 2.0 m high holding the 2,811 l culture would cover 1.4 square meters. The 784 l P. marina culture would require an additional 0.392 square meters if it were in a 2.0 m high tank. The floor space devoted to raising food for the bivalves in a BCS based hatchery is 26% of the floor space of an algal based hatchery.

Many operating and construction costs for a bivalve hatchery may be reduced by using BCS as a nutrient source. The cost of illuminating algal cultures is eliminated, as discussed in section IV. Algal culture vessels must be transparent, a requirement which severely limits the construction materials available. A variety of cheap materials are available for constructing opaque tanks which can be used for heterotrophic organisms. The small volumes of culture required by

heterotrophic organisms would reduce costs associated with the pumping, filtering, and heating of seawater as well as costs for tank construction and maintenance. In addition, certain economies of scale will be gained by use of a single large tank as opposed to using numerous 80 l vessels. Heterotrophic cultures require more aeration than algal cultures, but aeration costs may not be less in algal cultures because aeration is often used to provide mixing for homogeneous illumination.

The principal disadvantage to using BCS in a bivalve hatchery is the slow growth of oysters fed P. vestita compared to I. suecica. A hatchery using BCS and having oysters grow 2.1% per day will require a standing stock of 1,575 kg of oysters to achieve the same production as 1,105 kg of oysters fed I. suecica and growing 3.5% per day. An algal based hatchery, thus, requires only 70% of the standing stock of the BCS based hatchery. Costs related to maintaining the oyster stock will be less for an algal based hatchery. Costs associated with pumping seawater, maintenance and labor as well as the initial construction costs will all be reduced.

While this study has demonstrated that it is possible to raise juvenile oysters on heterotrophic microorganisms cultured on BCS, the question of whether oysters can be raised economically on such a diet remains to be answered. As a means of recycling nutrients from brewery wastes, it is very inefficient. Heterotrophic microorganisms raised on BCS may have the potential to lower a bivalve hatchery's operating costs. Many questions, both scientific and economic, which are not addressed in this study, must be answered, before BCS can be used successfully in a commercial venture. A pilot study, on a scale closer

to a commercial hatchery, may be the appropriate means to address these questions. In addition, further research into finding species and combinations of species of heterotrophic flagellates, which are better bivalve foods, may significantly improve the economics of operating a bivalve hatchery.

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Appendix A

Colony Characteristics and Cellular Morphology of

Bacterial Isolates Capable of Growth on BCS

isolate	colony shape	colony size (mm)	color	margin	elevation	cell shape	cell size (microns)	motility
3231	round	0.5	white	smooth	flat	coccobacilli	1.0 X 0.5	+
3233	round	1.0	white	smooth	flat	coccobacilli	1.0 X 0.7	+
3234	oval	5.0	white, dark center	wavy	convex	bacilli	1.2 X 0.3	+
3235	round	1.0	white	smooth	flat	bacilli	1.2 X 0.3	+
3236	irregular	4.0	yellow	smooth	raised	bacilli	1.0 X 0.2	-
5012	round	1.0	orange, white edge	smooth	flat	bacilli	1.0 X 2.0	+
5013	round	3.0	white,translucent	slighty irregular	flat	cocci, tetrads	0.5	-
5015	round	1.0	yellow	finely irregular	raised	bacilli	1.0 X 0.5	-
8121	round	5.0	white, dark center	scalloped	raised	bacilli	1.0 X 0.5	+
8122	round	2.0	white, pink center	finely scalloped	flat	bacilli	2.5 X 0.3	-
8123	round	7.0	white, pink center	scalloped	raised	bacilli	1.0 X 0.5	+
8124	round	7.0	white, pink center	scalloped	raised	bacilli	1.0 X 0.5	+
8125	round	4.0	white	slightly irregular	flat	bacilli	1.0 X 0.4	-
8126	round	3.0	white, pink center	scalloped	raised	bacilli	1.2 X 0.4	-

VITA

David S. Gussman

Born in Philadelphia, Pennsylvania, 22 February 1953. Graduated from Central High School in 1970. Earned a B.A. in Biology from Temple University in 1974. Received M.S. in Ecology from The Pennsylvania State University in 1977. Entered doctoral program in College of William and Mary, School of Marine Science in 1979.